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Mgr. Tomáš Pánek

Diverzita a evoluce anaerobních heteroloboseí

Diversity and evolution of anaerobic heteroloboseans

DISERTAČNÍ PRÁCE

Vedoucí závěrečné práce: doc. RNDr. Ivan Čepička, Ph.D.

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PODĚKOVÁNÍ

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PROHLÁŠENÍ AUTORA PRÁCE

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 28.06.2015

Mgr. Tomáš Pánek

PROHLÁŠENÍ O PODÍLU NA PUBLIKACÍCH

Výsledky prezentované v dizertační práci Mgr. Tomáše Pánka jsou společným dílem pracovníků Laboratoře diverzity a evoluce anaerobních protist a spolupracujících týmů. Podíl T. Pánka na jednotlivých publikacích je specifikován níže.

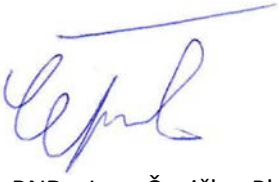
Pánek, T., Silberman, J.D., Yubuki, N., Leander, B.S., Cepicka, I. (2012) Diversity, evolution and molecular systematics of the Psalteriomonadidae, the main lineage of anaerobic/microaerophilic heteroloboseans (Excavata: Discoba). Protist 163: 807-831.

Podíl T. Pánka: Získání převážné většiny izolátů a jejich převedení do kultur, získání převážné většiny sekvenčních dat a jejich analýza, morfologická charakterizace jednotlivých linií. Sepisování a úprava rukopisu.

doc. RNDr. Ivan Čepička, Ph.D.

Pánek, T., Ptáčková, E., Čepička, I. (2014a). Survey on diversity of marine/saline anaerobic Heterolobosea (Excavata: Discoba) with description of seven new species. International Journal of Systematic and Evolutionary Microbiology 64: 2280-2304.

Podíl T. Pánka: Izolace a kultivace části publikovaných izolátů, získání některých sekvencí SSU rDNA, ultrastrukturní studie, sepisování a úprava části rukopisu.



doc. RNDr. Ivan Čepička, Ph.D.

Pánek, T., Simpson, A.G.B., Hampl, V., Čepička, I. (2014b). *Creneis carolina* gen. et sp. nov. (Heterolobosea), a novel marine anaerobic protist with strikingly derived morphology and life cycle. Protist 165: 542-567.

Podíl T. Pánka: Získání sekvenčních dat, morfologická charakterizace, velká část ultrastrukturní studie, sepisování a úprava rukopisu.

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Pánek, T., Táborský, P., Pachiadaki, M.P., Hroudová, M., Vlček, Č., Edgcomb, V.P., Čepička I. Combined culture-based and culture-independent approaches provide insights into diversity of jakobids, extremely plesiomorphic eukaryotic lineage. Rukopis je připraven k zaslání do tisku.

Podíl T. Pánka: Koordinace prací, fylogenetické analýzy, transmisní elektronová mikroskopie, sepisování a úprava rukopisu. Částečně též kultivace, získání sekvenčních dat a vyhledávání v environmentálních knihovnách.

doc. RNDr. Ivan Čepička, Ph.D.

DECLARATION OF THE AUTHOR´S CONTRIBUTION

Pánek, T., Táborský, P., Pachiadaki, M.P., Hroudová, M., Vlček, Č., Edgcomb, V.P., Čepička, I. Combined culture-based and culture-independent approaches provide insights into diversity of jakobids, extremely plesiomorphic eukaryotic lineage. Manuscript is ready for submission.

Contribution of T. Pánek: Research coordination, phylogenetic analyses, transmission electron microscopy, writing of the manuscript. Partially also cultivation, sequencing and searching in environmental libraries.

Dr. Virginia Edgcomb

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OSNOVA

ABSTRAKT

Tato práce se zaměřuje na fylogenezi, diverzitu a buněčnou strukturu obligátně anaerobních zástupců kmene Heterolobosea (Excavata: Discoba). S použitím kultivačních přístupů odhaluje jejich skrytou druhovou diverzitu a prezentuje 11 nově popsaných druhů. Pomocí fylogenetických analýz ukazuje, že obligátně anaerobní linie heterolobosеí se vyvinuly nejméně dvakrát nezávisle na sobě (Creneidae and Psalteriomonadidae). Čeleď Psalteriomonadidae je možno označit za hlavní anaerobní linii skupiny Heterolobosea, neboť obsahuje drtivou většinu známých obligátně ananerobních zástupců (16 druhů a 5 rodů). Většina druhů skupiny Psalteriomonadidae má akristátní mitochondrie, ačkoliv *Pseudoharpagon pertyi* má zřejmě zachovány ještě zbytky mitochondriálních krist. Buněčná struktura a životní cyklus *Creneis carolina*, samostatné anaerobní linie blízce příbuzné linii Percolatea, je unikátní. Architektura jejího bičíkatého aparátu není jednoduše porovnatelná s jinými eukaryoty, neboť u *C. carolina* došlo k unikátním přeskupením cytoskeletárních elementů. Anaerobní Heterolobosea jsou jen vzácně detekována pomocí environmentálních, na kultivaci nezávislých metod. Z toho důvodu je velmi obtížné odhadovat jejich skutečnou druhovou diverzitu. Naše nejnovější data o anaerobních jakobidech, další linii patřící mezi Discoba (Excavata), nicméně indikují, že kultivační přístup je poměrně úspěšný v odhalování druhové diverzity anaerobních exkavát. *In silico* analýza anaerobního energetického metabolismu naznačuje, že některé geny byly u anaerobních heterolobosеí získány horizontálním genovým přenosem nezávisle na sobě (ACS), zatímco některé další byly zděděny od společného předka podkmene Tetramitia (PPDK, Ppi-PFK, [FeFe]-hydrogenáza a její maturázy).

ABSTRACT

This thesis is focused on the phylogeny, diversity, and cell structure of obligately anaerobic Heterolobosea (Excavata: Discoba). Using culture-based approach, we have discovered their hidden species diversity and described 11 new species. Our phylogenetic analyses showed that obligately anaerobic heterolobosean evolved at least twice independently (Creneidae and Psalteriomonadidae). Psalteriomonadidae is a major anaerobic lineage of Heterolobosea as it includes 16 species and 5 genera. Most psalteriomonadids have acristate mitochondria, although *Pseudoharpagon pertyi* probably possesses remnants of mitochondrial cristae. Creneidae are represented just by a single species, *Creneis carolina*, which displays unique cell structure and life cycle. Architecture of its flagellar apparatus is not readily comparable with any other eukaryotes and at least some cytoskeletal elements have undergone unprecedented evolutionary positional changes. Anaerobic heterolobosean are just rarely detected by environmental, culture-independent approaches. Thus, it is impossible to estimate their real species diversity. Nevertheless, our current data on anaerobic jakobids, another lineage of Discoba, indicates that the culture-based approach is relatively powerful to discover species diversity of anaerobic excavates. *In silico* analysis of anaerobic energy metabolism indicates that some genes have been acquired by horizontal gene transfer independently in anaerobic heterolobosens (ACS), while some have been inherited from the common ancestor of the subphylum Tetramitia (PPDK, Ppi-PFK, [FeFe]-hydrogenase and its maturases).

1. Seznam publikací

Tato dizertační práce je založena na níže uvedených článcích publikovaných nebo (v jednom případě) připravených k zaslání do impaktovaného časopisu. V textu práce jsou citovány stejným způsobem jako ostatní použitá literatura, jsou ale vyznačeny podtržením.

1. **Pánek, T.**, Silberman, J.D., Yubuki, N., Leander, B.S., Cepicka, I. (2012) Diversity, evolution and molecular systematics of the Psalteriomonadidae, the main lineage of anaerobic/microaerophilic heteroloboseans (Excavata: Discoba). Protist 163: 807-831.

2. **Pánek, T.**, Ptáčková, E., Čepička, I. (2014a). Survey on diversity of marine/saline anaerobic Heterolobosea (Excavata: Discoba) with description of seven new species. International Journal of Systematic and Evolutionary Microbiology 64: 2280-2304.

3. **Pánek, T.**, Simpson, A.G.B., Hampl, V., Čepička, I. (2014b). *Creneis carolina* gen. et sp. nov. (Heterolobosea), a novel marine anaerobic protist with strikingly derived morphology and life cycle. Protist 165: 542-567.

4. **Pánek, T**., Táborský, P., Pachiadaki, M.P., Hroudová, M., Vlček, Č., Edgcomb, V.P., Čepička I. (zasláno k recenzi). Combined culture-based and culture-independent approaches provide insights into diversity of jakobids, extremely plesiomorphic eukaryotic lineage.

V **příloze disertační práce** lze nalézt některé dosud nepublikované výsledky týkající se analýzy transkriptomických dat heteroloboseí (odkazují na ně kapitoly 4.4 a 4.8 této práce). Kromě toho jsou součástí příloh i další tři publikované odborné články a jedna kapitola v knize, jichž jsem spoluautorem, ale které jsem do této práce nezahrnul.

2. Úvod

Ačkoliv už neplatí kdysi široce přijímaná představa, že někteří anaerobní prvoci reprezentují primárně amitochondriální linie, má výzkum anaerobů stále velký potenciál při studiu některých klíčových procesů a jevů v evoluci eukaryot. Dříve byl výzkum těchto organismů omezen na parazitické linie, v posledních letech se výraznější pozornost konečně věnuje i neparazitickým a volně žijícím zástupcům.

Anaerobní eukaryota v evoluci vznikla mnohokrát nezávisle na sobě a v různých podobách. Je proto potěšující, že se zlevňováním a zdokonalováním molekulárně-biologických metod se zvyšuje i počet podrobně zkoumaných linií. Velmi rychle se díky tomu rozšiřují naše znalosti o fungování mitochondriálních derivátů přizpůsobených činnosti v prostředí s nedostatkem kyslíku (*MROs*, zkratka z anglického výrazu *mitochondrion-related organelles*). To z anaerobů činí vynikající model pro studium adaptace eukaryot na život v extrémních[1] podmínkách. Tyto znalosti přispívají také k pochopení evoluce eukaryotické buňky a nelze opomenout ani jejich význam při výzkumu adaptace k endobiotickému a posléze i parazitickému způsobu života.

Fakt, že neznáme žádná primárně amitochondriální eukaryota[2], ovšem automaticky neznamená, že by některé konkrétní anaerobní skupiny nemohly ve skutečnosti reprezentovat hluboké nebo plesiomorfní eukaryotické linie. Kořen eukaryotického stromu totiž nebyl doposud odhalen, ačkoliv na toto téma existuje celá řada hypotéz (např. Derelle *et al*. 2015; He *et al*. 2015; Rogozin *et al*. 2009; Stechmann a Cavalier-Smith 2003).

Některé anaerobní skupiny dříve považované za primárně amitochondriální eukaryota, tedy řazené mezi dnes již neeexistující skupinu Archezoa (přehled viz Keeling 1998), našly svůj nový domov v superskupině Excavata. Ta, přestože obsahuje jen něco přes 0,1 % všech popsaných druhů eukaryot[3], se dnes dostává do centra zájmu při hledání plesiomorfních znaků a kořene eukaryotického stromu. Ukazuje se totiž, že malawimonády a jakobidi pravděpodobně představují, alespoň z hlediska architektury bičíkatého aparátu, nejvíce plesiomorfní linie eukaryot (O´Kelly a Nerad 1999; Simpson a Patterson 2001; Yubuki a Leander 2013). Jakobidi mají také nejplesiomorfnější mitochondriální genom (Burger *et al*. 2013; Lang *et al*. 1997). Je dokonce možné,

[1] Je možné polemizovat o tom, jestli je prostředí bez kyslíku pro eukaryotické organismy skutečně extrémní. Z pohledu člověka a většiny druhů eukaryot jsou opravdu anoxické habitaty extrémním prostředím. Někteří vědci ale zastávají názor, že eukaryota byla původně schopna žít bez kyslíku (Martin 2011) a tuto schopnost druhotně a mnohokrát nezávisle na sobě ztratila. Vedení polemiky v této otázce však není účelem mojí dizertační práce a ponechávám ji jiným autorům.

[2] Tato skupina se označovala jako Archezoa

[3] Odhad založen na datech prezentovaných v pracích Adl *et al*. (2007) a Pawlowski *et al*. (2012)

že ve své klasické podobě je skupina Excavata parafyletická a předek dnešních linií eukaryot připomínal svou morfologií právě jakobidy (Derelle *et al*. 2015).

Tématem mé dizertační práce jsou anaerobní zástupci kmene Heterolobosea, jedné z dosud málo studovaných skupin exkavát. Tento kmen v dřívější představě Cavaliera-Smithe zastával privilegovanou pozici na pomezí archezoí a ostatních eukaryot (např. Cavalier-Smith 1993). K vyslovení této hypotézy jej vedly poznatky o jednom z prvních studovaných anaerobních heterolobosei, druhu *Psalteriomonas lanterna*. Dříve se totiž myslelo, že v buňkách *P*. *lanterna* se vyskytují jak hydrogenosomy, tak modifikované mitochondrie (Broers *et al*. 1990). Dnes tyto dvě struktury považujeme za odlišné formy téže organely (de Graaf *et al*. 2009).

Anaerobní zástupci skupiny Heterolobosea byli jen velmi málo studováni, i když se vědělo o existenci několika anaerobních druhů řazených dokonce do různých rodů a čeledí. Hlavní otázkou řešenou v této práci je proto objasnění příbuzenských vztahů mezi anaerobními zástupci heterolobosei, odhalení jejich diverzity a vytipování druhů využitelných v následných transkriptomických a genomických studiích. Pokud by se potvrdilo, že anaerobní způsob života u heterolobosei vznikl několikrát nezávisle na sobě a v různých formách, byla by tato skupina ideální model pro studium evoluce anaerobního způsobu života.

Během doktorského studia se mi navíc podařilo získat řadu izolátů, sekvenčních a morfologických dat z jiných ananerobních eukaryotických linií. Některé z těchto vedlejších výsledků se pak staly součástí dalších publikací, které jsem nezahrnul do dizertační práce, ale přikládám je v příloze k nahlédnutí. Do dizertační práce jsem se naproti tomu rozhodl začlenit jeden současný projekt, který je zaměřen na odhalení diverzity anaerobních jakobidů (Excavata: Discoba) pomocí kombinace kultivačních a na kultivaci nezávislých metod (Pánek *et al.,* připraveno k zaslání do tisku). Anaerobní heterolobosea, podobně jako některé další anaerobní linie, nejsou v environmentálních knihovnách příliš zastoupena a náš vhled do jejich diverzity je tedy prozatím závislý hlavně na kultivačním přístupu. Jakobidi jsou naproti tomu jednou z nejpočetnějších linií exkavát v environmentálních knihovnách z anoxických prostředí a je tak možné díky studiu anaerobních jakobidů porovnat schopnost kultivačních a environmentálních metod odhalovat diverzitu anaerobních prvoků.

V textu práce zavádím české ekvivalenty některých názvů vyšších taxonů, které doposud nemají jiné používané české označení. Jedná se o tyto skupiny: Archezoa (archezoa, 2. p. archezoí); Excavata (exkaváti, 2. p. exkavát); Heterolobosea (heterolobosea, 2. p. heterolobosei); Jakobida (jakobidi, 2. p. jakobidů); Malawimonadea (malawimonády, 2. p. malawimonád). Ačkoliv jsem v poděkování přechyloval ženská jména mých zahraničních spolupracovníků, v textu práce ženská jména ponechám v originální podobě, tedy bez přechylování.

3. Cíle práce

1) Provést taxonomickou revizi dosud známých anaerobních druhů řazených mezi Heterolobosea, získat nové izoláty anaerobních heteroloboseí, provést jejich základní morfologickou charakterizaci a popis nových taxonů.

2) Rozřešit příbuzenské vztahy mezi jednotlivými liniemi anaerobních heteroloboseí pomocí molekulárních markerů, zejména SSU rDNA.

3) Charakterizovat životní cyklus u anaerobních heteroloboseí.

4) Charakterizovat struktury bičíkatého aparátu reprezentativních zástupců jednotlivých anaerobních linií, sjednotit terminologii užívanou pro jednotlivé elementy bičíkatého aparátu u heteroloboseí.

5) Prozkoumat morfologii mitochondriálních derivátů anaerobních heteroloboseí pomocí transmisní elektronové mikroskopie.

4. Výsledky práce v kontextu současného poznání

4.1. Heterolobosea – základní seznámení

Heterolobosea[4] patří mezi Excavata, konkrétně do linie Discoba. Ta zahrnuje také skupiny Jakobida, Euglenozoa a Tsukubamonadida (Derelle *et al*. 2015; Hampl *et al*. 2009; Kamikawa *et al*. 2014; Yabuki *et al*. 2011).

Monofylie skupiny Heterolobosea je podporována multigenovými a fylogenomickými analýzami, byť jsou v současnosti k dispozici data jen z několika málo druhů heteroloboseí, které navíc nepředstavují reprezentativní zástupce pokrývající všechny hlavní linie (např. Derelle *et al*. 2015; Hampl *et al*. 2009; Kamikawa *et al*. 2014; Rodríguez-Ezpeleta *et al*. 2007). Studium vnitřní fylogeneze heteroloboseí je založeno zejména na analýze SSU rDNA (např. Brown *et al*. 2012; Harding *et al*. 2013; Pánek *et al*. 2012, 2014b; Yubuki a Leander 2008).

Zástupci kmene Heterolobosea jsou heterotrofní, převážně volně žijící prvoci. Jsou však známé i fakultativně parazitické druhy, které jsou příležitostnými patogeny člověka či jiných živočichů – některé druhy rodu *Naegleria* a *Paravahlkampfia francinae* (více např. Butt 1966; De Jonckheere 2002; Visvesvara *et al*. 2009). Nejznámější a nejstudovanější z těchto patogenů je bezpochyby *Naegleria fowleri* (Butt 1966; Fowler a Carter 1965; John 1982; Marciano-Cabral 1988). Kromě toho byly některé další druhy heteroloboseí izolovány z rohovky pacientů trpících keratitidou (Aitken *et al*. 1996; Alexandrakis *et* al. 1998; Dua *et al*. 1998) nebo ze střev, žaber a dalších orgánů živočichů (Brugerolle a Simpson 2004; Dyková *et al*. 2001, 2006; Schuster *et al*., 2003). Patogenita těchto druhů nebyla dostatečně prokázána. Je ale jasné, že některé z nich jsou přinejmenším fakultativně endobiotické (např. *Percolomonas sulcatus* – Brugerolle a Simpson 2004, *Paravahlkampfia* sp. – Schuster *et al*. 2003).

V životním cyklu řady heteroloboseí se vyskytují tři životní stádia – améba (měňavka), bičíkovec a cysta. Přestože tento komplexní životní cyklus byl pozorován jen u některých druhů, zdá se, že jde o plesiomorfní stav u celého kmene Heterolobosea (Harding *et al*. 2013). Zástupci čeledi Acrasidae jsou jedinými známými exkaváty, kteří dokázali vytvořit ve svém životním cyklu i mnohobuněčné stádium, byť jde jen o velmi jednoduchou plodničku vznikající shlukováním buněk (viz např. Brown *et al*. 2012).

Z hlediska popsané druhové diverzity jsou Heterolobosea malou, nepočetnou skupinou. Doposud bylo popsáno pouze 150 druhů, navíc téměř třetinu z nich představují druhy rodu *Naegleria*. Ten, jak bylo řečeno výše, je významný z medicínského hlediska, a proto se mu věnuje

[4] Cavalier-Smith a jeho spolupracovníci dodnes používají pro skupinu stejného složení jméno Percolozoa (např. Cavalier-Smith 1991, 1993, 2003; Cavalier-Smith a Nikolaev 2008; Ruggiero *et al*. 2015).

relativně veliká pozornost. U druhu *Naegleria gruberi* byl osekvenován a podrobně analyzován i jaderný a mitochondriální genom (Fritz-Laylin *et al*. 2010; Opperdoes *et al*. 2011).

Zástupci kmene Heterolobosea obývají velmi širokou škálu různých habitatů. Můžeme je nalézt v půdě, sladkých vodách, termálních pramenech, oceánech, v extrémně slaných i extrémně kyselých prostředích nebo v útrobách živočichů (souhrn např. Pánek a Čepička 2012). Z hlediska podílu na celkové druhové diverzitě jsou Heterolobosea obzvláště významnou složkou společenstev z prostředí s extrémně vysokou salinitou, kde tvoří zhruba třetinu všech heterotrofních druhů eukaryot (Park *et al*. 2007, 2009; Park a Simpson 2011). V poslední době se ukazuje, že Heterolobosea mohou tvořit dokonce dominantní složku bakteriovorů v určitých typech extrémních habitatů (Reeder *et al*. 2015).

4.2. Diverzita anaerobních heterolobosei

Z anoxických a mikrooxických prostředí bylo v minulosti (před zahájením našeho výzkumu) popsáno několik druhů prvoků řazených do skupiny Heterolobosea[5], konkrétně šlo o *Psalteriomonas lanterna* (Broers *et al*. 1990; Psalteriomonadidae), *Sawyeria marylandensis* (O'Kelly *et al*. 2003; rod nezařazen do konkrétní čeledi), *Monopylocystis visvesvarai* (O'Kelly *et al*. 2003; rod nezařazen do konkrétní čeledi); *Lyromonas vulgaris* (syn. *Psalteriomonas vulgaris*) (Broers *et al*. 1993; Cavalier-Smith 1993; Lyromonadidae); *Percolomonas descissus* (syn. *Tetramitus descissus*) (Klug 1936; Larsen a Patterson 1990; Perty 1852; Percolomonadidae); *Vahlkampfia anaerobica* (Smirnov a Fenchel 1996; Vahlkampfiidae) a *Pleurostomum flabellatum* (Park *et al*. 2007; Ruinen 1938; rod nezařazen do konkrétní čeledi[6]). Není jisté, zda poslední jmenovaný druh je skutečně obligátně anaerobní. Je možné ale konstatovat, že obývá hypoxické prostředí s třetinovou koncentrací kyslíku v porovnání s normální mořskou vodou a na dostupných mikrofotografiích struktury mitochondrie nejsou patrné žádné kristy (Park *et al*. 2007). Ze tří z uvedených druhů (*Percolomonas descissus*, *Vahlkampfia anaerobica*, *Lyromonas vulgaris*) neexistovala před zahájením této práce žádná sekvenční data, tyto druhy byly navíc řazeny do tří různých čeledí (Percolomonadidae, Vahlkampfiidae, Lyromonadidae).

[5] Uváděné druhy je možné považovat za obligátně anaerobní/mikroaerofilní s jedinou potenciální výjimkou (*Pleurostomum flabellatum*). Vedle toho ale existuje několik dalších druhů, které obývají kyslíkem chudá prostředí a lze o nich uvažovat jako o fakultativních anaerobech. Především chci zmínit druh *Percolomonas sulcatus*, který žije ve střevě pulců ropuchy obecné a má diskovité mitochondriální kristy (viz Brugerolle a Simpson 2004) a druh *Naegleria gruberi*, která má vedle klasického aerobního metabolismu i některé enzymy typické pro anaerobní metabolimus (viz Fritz-Laylin *et al*. 2010; Tsaousis *et al*. 2014). Bylo by třeba také prostudovat dalších pět známých druhů rodu *Pleurostomum*, neboť obývají podobné prostředí jako *P. flabellatum* a mohou proto být též přizpůsobeni životu v nízké koncentraci kyslíku (přehled všech druhů rodu *Pleurostomum* viz Park *et al*. 2007). Stejně tak je pravděpodobné, že i další druhy rodu *Naegleria* budou obsahovat některé enzymy typické pro anaerobní metabolismus. Dalo by se spekulovat i o tom, že některé tyto enzymy získala obligátně a fakultativně anaerobní heterolobosea od společného předka.

[6] Rod *Pleurostomum* byl nedávno přeřazen do nově vytvořené čeledi Tulamoebidae (Kirby *et al*. 2015)

My jsme publikovali 48 nových izolátů anaerobních heteroloboseí představujících 15 druhů, z toho 11 jsme nově objevili a formálně popsali v souladu s mezinárodními pravidly zoologické nomenklatury (ICZN). Ukázali jsme, že všechny dříve popsané anaerobní druhy s výjimkou *Pleurostomum flabellatum* tvoří monofyletickou anaerobní linii, v našem pojetí čeleď, pro kterou jsme na základě pravidla priority zvolili jméno Psalteriomonadidae (Pánek *et al*. 2012). Do této čeledi patří i téměř všechny nově objevené a popsané druhy (Pánek *et al*. 2012, 2014a). Výjimkou je pouze druh *Creneis carolina*, který jsme zařadili do nové čeledi Creneidae (Pánek *et al*. 2014b). *Creneis carolina* má oproti příslušníkům čeledi Psalteriomonadidae i ostatním heteroloboseím řadu specifik, o nichž bude řeč dále. Souhrn všech v současnosti popsaných druhů čeledi Psalteriomonadidae a Creneidae včetně těch popsaných v našich publikacích lze nalézt v **tabulce 1**.

Dřívější systém anaerobních heteroloboseí jsme uvedli do souladu s našimi poznatky. Rody *Monopylocystis* a *Sawyeria* jsme zařadili do čeledi Psalteriomonadidae. Přesunuli jsme druh *Lyromonas vulgaris* do rodu *Psalteriomonas*, do něhož byl paradoxně původně při svém popisu zařazen (Broers *et al*. 1993). Jméno monotypického rodu *Lyromonas* a monotypické čeledi Lyromonadidae se tudíž stalo subjektivním synonymem rodu *Psalteriomonas* a čeledi Psalteriomonadidae (Pánek *et al*. 2012). Dále jsme druh *Vahlkampfia anaerobica* přesunuli do rodu *Monopylocystis* a druh *Percolomonas descissus* jsme z rodu *Percolomonas* a čeledi Percolomonadidae přesunuli do nově vytvořeného rodu *Harpagon* a do čeledi Psalteriomonadidae (Pánek *et al*. 2012).

Při popisu nových taxonů jsme vycházeli z důkladného porovnání morfologických charakteristik nových izolátů s již publikovanými daty. Naše závěry jsme vždy konfrontovali s výsledky fylogenetických analýz založených na SSU rDNA. Dbali jsme na vytváření monofyletických taxonů. Jako pomocné kritérium jsme používali též molekulární polymorfismus v sekvenci SSU rDNA. Genetická vzdálenost (p-distance po odstranění intronů) mezi jednotlivými druhy daných rodů čeledi Psalteriomonadidae byla v porovnání s mnoha jinými skupinami prvoků relativně vysoká. Například u rodu *Pseudoharpagon* se pohybovala v rozmezí 22,8 % – 26,9 %; u rodu *Harpagon* mezi 21,5 % a 21,8 % a v rámci rodu *Monopylocystis* se druhy lišily v 5,6 % – 19,2 % (Pánek *et al*. 2014a).

Taxon	Popis taxonu	Životní cyklus				Nc (Nn)	Habitat				
		A	AF	F	C		FW	B	M	I	S
•• Creneidae	**Pánek *et al*. 2014b**										
• *Creneis*	**Pánek *et al*., 2014b**										
Creneis carolina	**Pánek *et al*., 2014b**		•	•		1(1)			•		
•• Psalteriomonadidae	Cavalier-Smith, 1993										
• *Psalteriomonas*	Broers *et al*., 1990										
Psalteriomonas lanterna	Broers *et al*., 1990	•		(•)		6(5)	•				
Psalteriomonas vulgaris	Broers *et al*., 1993			•		1(0)	•				
Psalteriomonas magna	**Pánek *et al*., 2012**	•				3(3)	•				
• *Sawyeria*	O´Kelly *et al*., 2003	•				4(3)	•				
Sawyeria marylandensis	O´Kelly *et al*., 2003	•				4(3)	•				
• *Monopylocystis*	O´Kelly *et al*., 2003										
Monopylocystis visvesvarai	O´Kelly *et al*., 2003	(•)		(•)	(•)	7(6)		•	•	•	
Monopylocystis anaerobica	Smirnov a Fenchel, 1996	•				1(0)		•			
Monopylocystis disparata	**Pánek *et al*., 2014a**	(•)		(•)	(•)	3(3)			•		•
Monopylocystis elegans	**Pánek *et al*., 2014a**	•		•	•	1(1)		•			
Monopylocystis minor	**Pánek *et al*., 2014a**	•		•	•	2(2)			•		
Monopylocystis robusta	**Pánek *et al*., 2014a**	•			•	1(1)					•
• *Harpagon*	**Pánek *et al*., 2012**										
Harpagon descissus	Perty, 1852			•		>12[7](11)	•				
Harpagon schusteri	**Pánek *et al*., 2012**			•		6[8](6)	•				
Harpagon salinus	**Pánek *et al*., 2014a**			•		1(1)				•	
• *Pseudoharpagon*	**Pánek *et al*., 2012**										
Pseudoharpagon pertyi	**Pánek *et al*., 2012**			•		2(2)		•	•		
Pseudoharpagon longus	**Pánek *et al*., 2014a**	•		•	•	1(1)		•			
Pseudoharpagon tertius	**Pánek *et al*., 2014a**	•		•		2(2)			•		

Tabulka 1. Souhrn popsaných taxonů čeledí Psalteriomonadidae a Creneidae s vyznačením základních charakteristik jejich životního cyklu a habitatu, který obývají. Vysvětlivky (uvedeny v pořadí zleva dle záhlaví tabulky): A – améba; AF – améboidní bičíkovec; F – bičíkovec; C – cysta; Nc – celkový počet publikovaných nálezů, Nn – počet izolátů publikovaných v našich pracích. FW - sladkovodní prostředí, B – brakické prostředí, M – moře, I – vnitrozemská slaniska; S – slaniska poblíž mořského pobřeží, avšak s vyšší salinitou. Černá tečka znamená přítomnost daného životního stádia nebo výskyt v daném

[7] Druh *Harpagon descissus* byl zaznamenán v celé řadě studií. Kromě Perty (1852) a Pánek *et al.* (2012) jej uvádí též Bernard *et al*. (2000), Brugerolle a Simpson (2004), Klug (1936) a Murase *et al*. (2014). Odlišení od druhu *H. schusteri* je však podle dostupných informací o těchto dalších nálezech obtížné a celkové číslo všech nálezů je třeba brát jen jako orientační. V případě práce Bernard *et al*. (2000) nelze dokonce vyloučit, že šlo ve skutečnosti o zástupce rodu *Pseudoharpagon*.

[8] *Harpagon schusteri* byl kromě práce Pánek *et al.* (2012) zaznamenán ještě ve studii Murase *et al*. (2014).

typu prostředí. Je-li tečka v závorce (•), indikuje to nepřítomnost daného životního stádia v některých kultivovaných izolátech sledovaného druhu.

4.3. Použitý druhový koncept, kritika molekulárního druhového konceptu

Při popisu nových druhů ve skupině Heterolobosea je velmi důležité identifikovat a řádně charakterizovat různá životní stádia zkoumaného izolátu a druhu, aby byly v budoucnu porovnatelné s jinými izoláty (druhy). Často se stává, že daný izolát v laboratorních podmínkách netvoří všechna stádia, je lépe proto druhy popisovat na základě znalostí získaných porovnáváním více izolátů. Porovnávání různých izolátů nám navíc umožňuje udělat si představu o variabilitě zvolených znaků v rámci druhů i mezi druhy.

V minulosti byla nejdůležitějším životním stádiem pro popis nových druhů cysta (Page 1988). Dnes by podle našeho přesvědčení u těch druhů, u kterých je známo bičíkaté stádium, měla být velká pozornost věnována právě morfologii bičíkovců. Bohužel se tak ale často neděje (např. De Jonckheere *et al*. 2011), i když bičíkovci poskytují obecně více taxonomicky významných morfologických znaků než stádium cysty nebo améby. Těchto znaků lze navíc využít nejen při popisu nových druhů, ale i rodů, čeledí apod. Morfologie bičíkovců kmene Heterolobosea je kromě toho i přímo porovnatelná s morfologií jiných exkavát.

V našich pracích jsme však dbali na co nejpodrobnější charakterizaci nejen bičíkovců, ale všech dostupných životních stádií daných druhů. Například rozlišení druhů rodu *Monopylocystis* jsme založili hlavně na stádiu améby (Pánek *et al.* 2014a). Jsem přesvědčen, že podrobná charakterizace všech dostupných životních stádií je při popisu nových druhů kmene Heterolobosea nezbytná, protože původní životní cyklus byl u řady linií modifikován vynecháním stádia améby či bičíkovce. Tento přístup zajistí nejvyšší možnou míru morfologického srovnání jednotlivých linií heteroloboseí mezi sebou. Některé práce popisující nové druhy bohužel na podrobnou charakterizaci morfologie rezignovaly (např. *Naegleria canariensis* a *N*. *tenerifensis* – De Jonckheere 2006).

Na základě porovnávání bičíkovců různých izolátů patřících do čeledi Psalteriomonadidae jsme jako taxonomicky významné znaky pro popis nových druhů vyhodnotili zejména tvar či délku buňky, přítomnost, průběh a délku potravní rýhy, počet bičíků v bičíkatém aparátu, relativní délku bičíků vůči sobě a vůči délce celé buňky. Druhy *Harpagon schusteri* a *H*. *descissus* jsme odlišili na základě rozdílné tvarové variability buněk (Pánek *et al.* 2012).

Améby kmene Heterolobosea jsou obvykle válcovité, poměrně rychle lezoucí buňky vybavené eruptivními lobopodiemi. Při popisu nových druhů na základě morfologie améb jsme využívali morfologie a pozice jádra, velikosti lokomotivní formy (délka buňky, poměr délky a šířky), přítomnost či tvar uroidu a vlečných filament, charakter rozhraní granuloplasmy a hyaloplasmy.

Determinaci některých druhů na základě morfologických znaků komplikuje fakt, že porovnání stádií bičíkovce a améby mezi sebou je v podstatě nemožné. Jeden z mála morfologických znaků, který bývá stabilní v rámci různých stádií životního cyklu, je morfologie jádra (např. pozice jadérek, rozmístění heterochromatinu apod.). Na úrovni druhu u čeledi Psalteriomonadidae jsou stabilní i nároky na salinitu (sladkovodní x mořské/brakické linie – viz **tabulka 1**). Pokud jde o znaky použitelné při popisu cyst daných druhů, nejdůležitější je velikost, tvar a způsob otevírání cysty (švy, póry apod.).

Přístup, který jsme zvolili pro popis druhů ve skupině Psalteriomonadidae, je relativně konzervativní a může podhodnocovat reálný počet druhů. Nicméně poměrně dobře odráží celkovou známou variabilitu, neboť vychází z podrobné charakterizace jednotlivých izolátů a hledání dostatečně stabilních, taxonomicky významných rozdílů. Dobrým příkladem možného podhodnocení druhové diverzity je *Pseudoharpagon pertyi*. Genetická vzdálenost (SSU rDNA) mezi dvěma izoláty řazenými do tohoto druhu je téměř 20 %. Je tudíž možné, ba dokonce pravděpodobné, že se v budoucnu podaří nalézt spolehlivé rozdíly opravňující rozdělení *P. pertyi* do několika druhů. K tomu bude ale potřeba získat a prozkoumat více izolátů patřících do tohoto potenciálního druhového komplexu.

Alternativou ke zvolenému konceptu popisu nových druhů kmene Heterolobosea je molekulární druhový koncept (De Joncheere 2004). Ten jsme však z několika důvodů odmítli v případě Psalteriomonadidae použít. Tento koncept dle našeho přesvědčení příliš nadhodnocuje počet druhů a obsahuje několik závažných nedostatků, na které jsme opakovaně v minulosti upozorňovali (Pánek a Čepička 2012; Pánek *et al*. 2012).

Molekulární druhový koncept původně vznikl jako odpověď na problém odlišení patogenních a nepatogenních druhů rodu *Naegleria*. Ty není možné jednoduše od sebe rozpoznat morfologicky, a proto je využíváno molekulárních markerů, v minulosti například allozymových vzorů (Robinson *et al*. 1992). De Jonckheere pak druhový koncept rodu *Naegleria* založil na odlišnostech v sekvenci ITS regionu (ITS1, ITS2 a 5.8S rDNA), přičemž sekvenční variabilitu konfrontoval s výsledky allozymových analýz (De Jonckheere 2004). Ve svých pozdějších publikacích však bez řádného zdůvodnění rozšířil tento druhový koncept na celou čeleď Vahlkampfiidae (De Jonckheere a Brown 2005). Při pohledu na **obrázek 1** je jasné, že takové zobecnění je přinejmenším velmi odvážné, neboť čeleď Vahlkampfiidae zahrnuje zástupce nejméně čtyř hlavních linií heterolobosei a netvoří monofylum. De Jonckheere považuje i rozdíl pouhých dvou záměn v sekvenci ITS2 (bez ohledu na polohu těchto záměn) za dostatečnou indikaci toho, že dané izoláty nepatří do stejného druhu (De Jonckheere 2004).

Hlavní komplikace tohoto konceptu lze ve zkratce shrnout do dvou bodů: (1) Bez relevantního zdůvodnění předpokládá u podstatné části kmene Heterolobosea jen minimální vnitrodruhový polymorfismus, nebere přitom v úvahu polohu jednotlivých záměn na molekule DNA (Pánek a Čepička 2012; Pánek *et al*. 2012). (2) Nepředpokládá vnitrogenomový polymorfismus v ITS

regionu, přestože u rodu *Naegleria* byl zaznamenán přímo v sekvenci ITS2 (až osm záměn; Dyková *et al*. 2006). Nepřímým negativním důsledkem používání tohoto konceptu je i praktická rezignace na podrobnou charakterizaci jednotlivých izolátů a druhů. Protože získání sekvence ITS regionu dostačuje k popisu druhu, není nutné důkladně morfologicky charakterizovat studované organismy a snažit se hledat další rozdíly (viz například De Jonckheere 2006, 2007).

Je třeba zdůraznit, že existuje výrazný rozdíl mezi identifikací již dříve popsaného druhu pomocí sekvence ITS2 regionu (nebo jiného dostatečně variabilního molekulárního markeru), tedy barcodingem, a popisem nového druhu pouze na základě rozdílu v sekvenci zvoleného markeru bez toho, aniž by existovaly dostatečné doklady k tvrzení, že se dané izoláty od sebe morfologicky, fyziologicky, ekologicky či biochemicky liší a reprezentují samostatné evoluční linie. Zatímco identifikace izolátu s pomocí molekulárního markeru je naprosto v pořádku, molekulární druhový koncept v jeho současné podobě nelze u heterolobosei akceptovat. Nyní se zdá, že i J. De Jonckheere se svými spolupracovníky konečně od použití molekulárního druhového konceptu v této podobě ustupuje (Geisen *et al*. 2015).

4.4. Taxonomie a fylogeneze skupiny Heterolobosea

Skupina Heterolobosea[9] byla původně vytvořena jako taxon sdružující dvě linie amébovitých organismů, totiž Schizopyrenida a Acrasida (Page a Blanton 1985). Její monofylii s použitím molekulárních dat poprvé prokázala fylogenetická analýza genu pro GAPDH pomocí sekvencí získaných z rodů *Naegleria* (Schizopyrenida) a *Acrasis* (Acrasida) (Roger *et al*. 1996). Od té doby se znalosti o diverzitě i disparitě skupiny značně rozšířily. V současnosti Heterolobosea zahrnují dva podkmeny – (1) plesiomorfní Pharyngomonada s jediným popsaným rodem *Pharyngomonas* a (2) druhově bohatá, morfologicky a ekologicky diverzifikovaná Tetramitia (Cavalier-Smith a Nikolaev 2008; Harding *et al*. 2013; Park a Simpson 2011). Vedle toho je třeba mít na paměti, že existují ještě nejméně dvě kandidátní linie, které mohou představovat další samostatné podkmeny heterolobosei – plesiomorfní bičíkovec *Percolomonas sulcatus*, z něhož doposud chybí sekvenční data (Brugerolle a Simpson 2004), a améba označovaná jako „BB2“, která ve fylogenetických analýzách založených na SSU rDNA tvoří sesterskou větev všem ostatním heterolobosem, ovšem s nízkou statistickou podporou (Harding *et al*. 2013).

Podobně jako u mnoha jiných skupin, je i u heterolobosei nejpoužívanějším molekulárním markerem SSU rDNA. Postupem času se podařilo získat sekvence tohoto genu z většiny důležitých linií, stal se tak hlavním zdrojem naší současné představy o jejich vnitřní fylogenezi (Brown *et al*.

[9] Skupině Heterolobosea přiznávám status kmene, podobně jako například Cavalier-Smith. Ten ale skupinu stejného složení nazývá Percolozoa (viz dále).

2012; Harding *et al*. 2013; O'Kelly *et al*. 2003; Pánek *et al*. 2012, 2014b; Park *et al*. 2012). Ukázalo se, že řada dřívějších předpokladů o příbuzenských vztazích (odvozených od podobnosti morfologie cyst nebo absence některých stádií v životním cyklu) byla chybná. Taxonomie skupiny se dostala do chaosu, který, i přes určité pokroky (např. Brown *et al*. 2012 – Acrasidae; Kirby et al. 2015 – Tulamoebidae; Pánek *et al*. 2012 – Psalteriomonadiae), pokračuje do dnešní doby. Jedním z příkladů tohoto chaosu je sběrná čeleď Vahlkampfiidae (viz **obrázek 1**).

Své vlastní alternativní pojetí systému heterolobosei vytvořil Cavalier-Smith a jeho spolupracovníci (např. Cavalier smith 1993; Cavalier-Smith a Nikolaev 2008), tento systém není ale většinou ostatních autorů používán. Cavalier-Smith dodnes používá pro skupinu stejného složení jméno Percolozoa (např. Cavalier-Smith 1991, 1993, 2003; Cavalier-Smith a Nikolaev 2008; Ruggiero *et al*. 2015). Původně tuto skupinu považoval za klíčovou v evoluci eukaryot, neboť měla reprezentovat přechod mezi primárně amitochondriální linií Archezoa a pokročilejšími eukaryoty (Cavalier-Smith 1993). Když se začaly kumulovat důkazy, že domněle amitochondriální prvoci nemusí reprezentovat nejhlubší linie eukaryot a mitochondrie byla nejspíš přítomna u předka všech známých eukaryotických linií (např. Embley a Hirt 1998; Roger *et al*. 1998, Tachezy *et al*. 2001), tak Cavalier-Smith od tohoto scénáře ustoupil (Cavalier-Smith 2003). Taxon Percolozoa v následujících verzích svého systému eukaryot nicméně zachoval a rozdělil jej na dva podkmeny (Tetramitia a Pharyngomonada). Podkmen Pharyngomonada přitom považoval za linii oddělenou ještě před vznikem stádia améby (Cavalier-Smith a Nikolaev 2008). Taxon Heterolobosea, který vytvořili Page a Blanton (1985) pro akrasidní hlenky (Acrasida) a válcovité améby schopné mnohdy i tvorby bičíkatého stádia (Schizopyrenida), v jeho systémech představuje jen jednu ze tří tříd podkmene Tetramitia. V tomto pojetí je s velkou pravděpodobností parafyletický. Podle mého názoru je označení Heterolobosea pro skupinu obsahující podkmeny Tetramitia, Pharyngomonada a linii BB2 v současnosti vhodnější. Jak bylo uvedeno výše, je totiž zřejmé, že stádium améby se v evoluci vytvořilo ještě před divergencí těchto tří linií (Harding *et al*. 2013).

Pokud budou objeveny linie příbuzné heterolobosem, které se oddělily ještě před vznikem améboidního stádia, doporučuji nezahrnovat je do taxonu Heterolobosea. Adoptovat jméno Percolozoa pro tuto skupinu bych ale považoval za nešťastné, neboť je odvozeno od názvu rodu *Percolomonas*, který amébovité stádium ztratil druhotně. V našich článcích jsme ze systému Cavaliera-Smithe převzali názvy podkmenů Tetramitia a Pharyngomonada, ačkoliv pro celý kmen používáme jméno Heterolobosea. Toto pojetí skupiny Heterolobosea a jejího základního členění je v souladu i s pozdější klasifikací eukaryot dle Adl *et al*. (2012), ti ovšem namísto Pharyngomonada používají název Pharyngomonadidae. V dalším textu tak bude jméno Heterolobosea používáno pro celý kmen, nikoliv jen pro jednu třídu podkmene Tetramitia.

Ačkoliv jsou fylogenetické analýzy SSU rDNA poměrně úspěšné v rozlišení příbuzenských vztahů uvnitř některých linií heterolobosei, hlubší vztahy rozlišit nedokáží. Není proto možné provést celkovou taxonomickou revizi, aniž bychom se vyhnuli riziku, že i nově vytvořený systém bude založen na parafyletických či polyfyletických taxonech. My jsme se rozhodli překlenout toto přechodné období rozdělením podkmene Tetramitia do sedmi linií, které lze na základě současných poznatků považovat za monofyletické, i když pro většinu z nich nelze stanovit synapomorfie a jejich vzájemné vztahy jsou zcela nejasné (Tetramitia I-VII; Pánek *et al.* 2014b – viz **obrázek 1**).

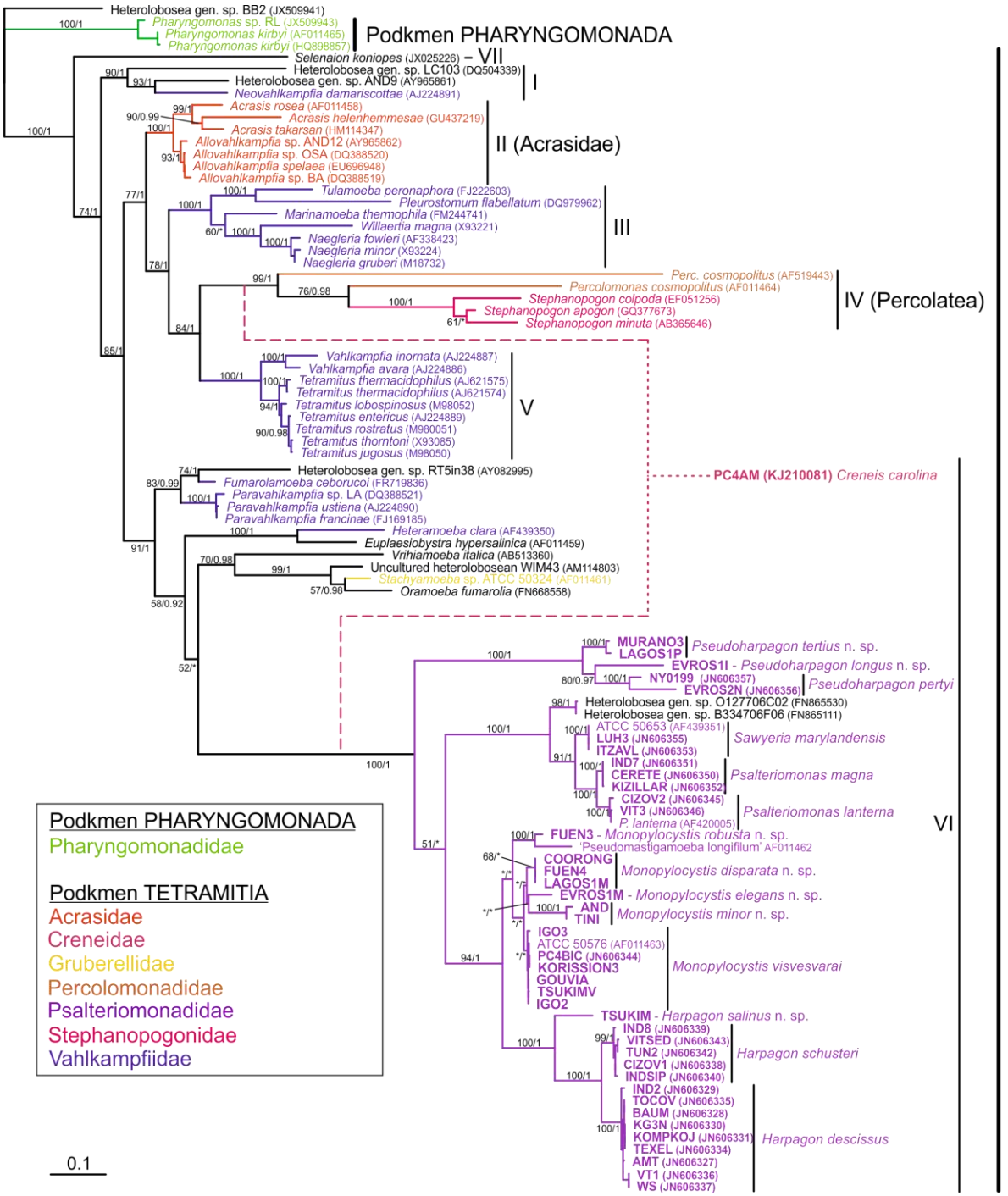
Z několika taxonů (*Naegleria gruberi*, *Stachyamoeba* sp. ATCC 50324, *Sawyeria marylandensis*, *Acrasis kona*) bylo získáno dostatek sekvenčních dat, aby mohly být zahrnuty do multigenových analýz (např. Hampl *et al*. 2009; He *et al*. 2014; Rodríguez-Ezpeleta *et al*. 2007). Na základě těchto analýz ovšem nelze rozhodnout o vzájemné příbuznosti jednotlivých výše zmíněných linií heterolobosei, a to hlavně díky nízkému počtu taxonů, které navíc nejsou dostatečně reprezentativní.

Nově získaná data			
Druh	**EST data**	**Genom**	**Životní styl**
Creneis carolina	454, Illumina	-	Obligátně anaerobní druh
Harpagon schusteri	454	-	Obligátně anaerobní druh
Heteramoeba clara	Illumina	-	Aerobní druh
Neovahlkampfia damariscottae	454	Draft	Aerobní druh
Sawyeria marylandensis	454	-	Obligátně anaerobní druh
Veřejně dostupná data			
Druh	**EST data**	**Genom**	**Životní styl**
Naegleria gruberi	Ano	Ano	Aerobní/ fak. anaerobní druh
Percolomonas cosmopolitus WS	Ano	-	Aerobní druh
Percolomonas cosmopolitus 2	Ano	-	Aerobní druh
Psalteriomonas lanterna	Ano*	-	Obligátně anaerobní druh
Stachyamoeba sp. ATCC 50324	Ano*	-	Aerobní druh

Tabulka 2. Přehled veřejně dostupných a nově získaných transkriptomických a genomických projektů heterolobosei. Hvězdička znamená, že je z daného organismu k dispozici jen velmi málo dat.

Během svého doktorského studia jsem se podílel na získání EST dat z několika doposud málo studovaných linií heterolobosei (příprava masových kultur, izolace RNA, z významné části i analýza získaných dat; přehled viz **tabulka 2**). Jedním z cílů tohoto projektu bylo zlepšení našich znalostí o fylogenezi heterolobosei. Bez znalosti vnitřní fylogeneze je totiž téměř nemožné vytvořit dostatečně podložené hypotézy o evoluci tohoto ekologicky i morfologicky nesmírně rozmanitého kmene eukaryot. Výsledek naší předběžné fylogenomické analýzy provedené ve spolupráci s Dr. Martinem Kolískem (University of British Columbia, Kanada) je uveden v příloze (**obr. S1**). Ačkoliv některé větve

heteroloboseí nebyly rozlišeny s dostatečnou statistickou podporou, je možné říci, že anaerobní čeledi Creneidae a Psalteriomonadidae, o nichž pojednává tato práce, si nejsou blízce příbuzné. *Neovahlkampfiadamariscottae* se větví sestersky ke všem ostatním studovaným heteroloboseím. Bohužel ale i tak chybí data k provedení fylogenomické analýzy zahrnující zástupce podkmene Pharyngomonada, liniii „BB2“, *Percolomonassulcatus* a TetramitiaII, V a VII.



← Obrázek 1. Současná taxonomie kmene Heterolobosea namapovaná na fylogenetický strom SSU rDNA. Barevně je vyznačena příslušnost dané linie ke konkrétní čeledi (viz legenda). Černě označené terminální větve nebyly doposud zařazeny do žádné čeledi nebo jde o environmentální sekvence. Přerušovanou čarou jsou naznačeny dvě možné fylogenetické pozice čeledi Creneidae dle Pánek *et al.* (2014b). Fylogenetický strom byl převzat z Pánek *et al.* (2014a) a upraven. Hodnoty uvedené u jednotlivých větví reprezentují bootstrapovou podporu maximální věrohodnosti a Bayesovu posteriorní pravděpodobnost.

4.5. Charakterizace životního cyklu anaerobních zástupců skupiny Heterolobosea

Jak bylo uvedeno v kapitole 4.1., již společný předek všech dnes známých zástupců skupiny Heterolobosea byl nejspíše schopen v životním cyklu tvořit všechna základní životní stádia, které známe u dnešních heteroloboseí - měňavku, bičíkovce a cystu (Harding *et al*. 2013). Při studiu dostupných informací o různých liniích heteroloboseí dojdeme navíc k závěru, že jak améba, tak bičíkovec byli původně schopni aktivně přijímat potravu. Centrální roli v životním cyklu přesto hrálo stádium améby. To bylo totiž schopno jak encystace, tak transformace na bičíkovce. V této původní podobě nalezneme životní cyklus například u druhu *Naegleria gruberi* (Fulton a Dingle 1967), *Oramoeba fumarolia* (De Jonckheere *et al*. 2011), *Heteramoeba clara* (Droop 1962), ale i mnoha dalších heteroloboseí včetně příslušníků rodu *Pharyngomonas* (Harding *et al*. 2013), kteří patří mezi nejhlubší známé linie kmene Heterolobosea (Cavalier-Smith a Nikolaev 2008; Harding *et al*. 2013; Park a Simpson 2011). U rodu *Naegleria* je tento životní cyklus ovšem poněkud modifikován. Bičíkovci většiny druhů tohoto rodu nejsou schopni přijímat potravu a dělit se, slouží jen jako dočasné transportní stádium (De Jonckheere 2002).

Tvorbu améby lze indukovat například kultivací na pevném živném médiu (De Jonckheere *et al*. 2011, Fulton a Dingle 1967; Harding *et al*. 2013). Transformaci améby na bičíkovce lze vyvolat přidáním tekutého média (*ibid.*) nebo jako odpověď na stres (změna osmolarity, teploty, hladovění) (Fulton 1977). Encystace je pak alternativní odpověď améby na působení stresových podmínek.

Zcela v souladu s výše uvedenými tvrzeními je fakt, že většina heteroloboseí, u nichž známe stádium améby, tvoří cysty. Naopak, chybí-li stádium améby, není obvykle známé ani stádium cysty. Jedinou výjimkou, o které vím, je linie zahrnující striktní bičíkovce schopné tvorby cyst – Percolatea (*Percolomonas cosmopolitus* a *Stephanopogon* spp.) (Fenchel a Patterson 1986; Lwoff 1936; Raikov 1969).

Psalteriomonadidae dnes představují, pokud jde o druhovou diverzitu a počet izolátů, jednu z nejprozkoumanějších linií heteroloboseí. Mohli jsme si proto dovolit zabývat se podrobněji i jejich životním cyklem. Na základě těchto dat můžeme říci, že společný předek čeledi Psalteriomonadidae byl schopen tvorby stádia améby, cysty i bičíkovce. Zřejmě i u Psalteriomonadidae platí centrální role

amébovitého stádia, které je (na rozdíl od bičíkovce) schopné encystace. To naznačuje fakt, že cysty nebyly nikdy pozorovány v kultuře, kde chybělo stádium améby, a to včetně rodu *Harpagon*, u něhož stádium améby pravděpodobně zcela chybí (je známo 20 izolátů, které byly kultivovány na různých živných médiích).

Je možné, že předek linie *Psalteriomonas* + *Sawyeria* zcela ztratil schopnost tvorby cyst, přestože si zachoval schopnost tvorby améby. Podobná situace je v rámci heteroloboseí poměrně vzácná. Protože nikdy nebyli pozorováni bičíkovci rodu *Sawyeria*, je možné, že i zde došlo k jejich ztrátě.

Přítomností/nepřítomností určitého životního stádia se ale lišily i izoláty uvnitř několika druhů psalteriomonadidů, konkrétně se jednalo o druhy *Monopylocystis disparata*, *M. visvesvarai* a *Psalteriomonas lanterna* (Pánek *et al.* 2012, 2014b). Izoláty obou druhů rodu *Monopylocystis* byly přitom kultivovány s použitím stejného živného média. O důvodech těchto odlišností mezi izoláty lze pouze spekulovat a nelze zcela vyloučit ani to, že jde o permanentní ztrátu schopnosti tvořit určité stádium. Osobně se ale domnívám, že to může být dáno přítomností různých druhů bakterií v kultuře. Vliv diety na zastoupení bičíkovců v kultuře byl v minulosti skutečně prokázán u druhu *Oramoeba fumarolia* (De Jonckheere *et al*. 2011).

Životní cyklus druhu *Creneis carolina* se zcela vymyká kanonickému životnímu cyklu heteroloboseí. *C. carolina*, jako jediný dosud známý zástupce kmene Heterolobosea, totiž tvoří stádium améby permanentně vybavené bičíkem (tj. améboidního bičíkovce). Navíc je schopen se transformovat do dalšího neobvyklého stádia, bizarního mnohobičíkovce vybaveného dvěma předními bičíky a 12 bičíky umístěnými po obvodu buňky (Pánek *et al.* 2014b).

4.6. Výzkum diverzity anaerobních exkavát pomocí metod nezávislých na kultivaci

Ačkoliv jsme při výzkumu diverzity anaerobních heteroloboseí používali výhradně klasické, na kultivaci založené metody, odhalili jsme překvapivě velikou druhovou diverzitu. Tyto metody jsou ale jen část arzenálu, který v současnosti při studiu diverzity protist lze využít. Další část tvoří metody na kultivaci nezávislé, jež odhalují obrovskou genetickou diverzitu protist snad ve všech typech habitatů (Pawlowski *et al*. 2012) a přináší důkazy o existenci řady doposud nekultivovaných linií prvoků (např. Bráte *et al*. 2010; Massana *et al*. 2014; Not *et al*. 2007). V kombinaci se stále se snižujícími finančními náklady masivního paralelního sekvenování znamená použití těchto environmentálních přístupů veliký pokrok v odhalování diverzity jednobuněčných eukaryot, jakož i v pochopení vztahů v rámci

mikrobiálních společenstev a determinant, které složení těchto společenstev formují (např. Lima-Mendez *et al*. 2015; Nemergut *et al*. 2013; Tedersoo *et al*. 2014).

Doposud publikované výsledky environmentálních studií nicméně neobsahují téměř žádné záznamy o přítomnosti heteroloboseí v anoxických vzorcích. Výjimkami jsou studie Amaral-Zettler *et al*. (2011) a Murase *et al*. (2014). První z nich odhalila přítomnost heteroloboseí v Río Tinto, extrémně kyselé řece ve Španělsku. Detailní fylogenetická analýza následně ukázala, že jde o nekultivovanou linii psalteriomonadidů, která je sesterská seskupení rodů *Sawyeria* + *Psalteriomonas* (Pánek *et al*. 2012). Druhá studie (Murase *et al*. 2014) testovala změny ve struktuře mikrobiálního společenstva půdy z rýžového pole vystavené různým podmínkám (různý obsah kyslíku a organické hmoty). Ukázalo se, že v anoxických vzorcích obohacených o organickou hmotu jsou významnou složkou mikrobiálního společenstva druhy *Harpagon descissus* a *H. schusteri*. Autoři nicméně upozorňují, že přítomnost těchto druhů ve vzorcích byla odhalena až s použitím nově navržených univerzálních primerů, které byly předem testovány, aby dobře detekovaly i Heterolobosea. Tyto výsledky naznačují, že by sladkovodní zástupci čeledi Psalteriomonadidae mohly hrát důležitou roli jako bakteriofágové v určitých typech půd bohatých na tlející rostlinnou biomasu.

V této souvislosti stojí za zmínku, že také v environmentálních knihovnách z hypersalinních prostředí Heterolobosea téměř chybí. To je v rozporu s mikroskopickými pozorováními, které ukazují, že Heterolobosea v těchto habitatech tvoří většinu pozorovaných morfotypů heterotrofních prvoků (Park a Simpson 2015). Nízké zastoupení v environmentálních studiích není případ pouze heteroloboseí, ale i jiných dobře známých anaerobních linií, z nichž řada patří mezi Excavata. To potvrdila například podrobná analýza environmentálních dat skupiny Fornicata (Kolisko *et al*. 2010). Absence těchto linií v environmentálních studiích je pravděpodobně arteficiální, neboť s použitím kultivačního přístupu jsou neustále izolovány nové linie nejen fornikát, ale i jiných anaerobních exkavát. To ostatně ukazuje i tato práce. Jednou z možností, jak se s tímto nedostatkem v budoucnu vyrovnat, je použití specifických primerů navržených tak, aby bylo možné úspěšně amplifikovat sekvence molekulárních markerů i z těchto linií.

Co se týká abundance a genetické diverzity, vévodí archivům environmentálních sekvencí zejména Alveolata, Stramenopiles a Rhizaria. Z exkavát jsou detekováni především zástupci skupin Euglenozoa (např. Edgcomb *et al*. 2011; Lara *et al*. 2009; Orsi *et al*. 2011) a Jakobida (např. Orsi *et al*. 2012; Stock *et al*. 2009; Weber *et al*. 2014). Zatímco Euglenozoa jsou druhově nejpočetnější skupinou exkavát, neboť představují ca 1500 z celkem 2500 popsaných druhů (Adl *et al*. 2007), jakobidi obsahují v současnosti jen 10 druhů, z nichž pouze jeden je anaerobní (*Andalucia incarcerata*; Simpson a Patterson 2001). Význam jakobidů navíc v posledních letech výrazně vzrůstá, ukazuje se, že by mohlo jít o nejvíce plesiomorfní skupinu eukaryot (Burger *et al*. 2013; Derelle *et al*. 2015; Yubuki a Leander 2013). Bohužel v případě anaerobních jakobidů existuje výrazný deficit v odhalování

jejich skutečné diverzity na straně tradičních, na kultivaci závislých přístupů. V jedné naší recentní studii jsme se proto rozhodli podrobně analyzovat dostupná sekvenční data z environmentálních studií, zkombinovat je s analýzou 21 nových izolátů anaerobních jakobidů a prozkoumat reálnou diverzitu, fylogenezi a ekologický význam této skupiny. Rukopis, který byl z výsledků tohoto projektu sepsán (Pánek *et al.*, připraveno k zaslání do tisku), jsem se rozhodl zahrnout do své dizertační práce, ačkoliv se přímo netýká heteroloboseí. Doplňuje totiž obraz toho, jakým způsobem lze v současnosti studovat diverzitu anaerobních prvoků a jaké jsou výhody a nevýhody jednotlivých v současnosti používaných metod. Na příkladu této zřejmě nejčastěji detekované skupiny anaerobních exkavát v environmentálních knihovnách ukazuje, že kultivační přístup je schopen odhalit významnou část její druhové diverzity.

Na závěr bych se rád letmo zmínil, že v současnosti už existují metody, které umožňují základní morfologickou charakterizaci, získání genomických či transkriptomických dat z jedné buňky izolované přímo z přírody. Ačkoliv tyto metody byly do praxe zavedeny nedávno, výrazně přispěly k hlubšímu poznání nekultivovatelných linií organismů (např. Lax a Simpson 2013; Rinke *et al*. 2013; Stepanauskas 2012; Yoon *et al*. 2011) a mají obrovský potenciál pro budoucnost. Co se týká heteroloboseí, mohou být využity například při výzkumu diverzity a metabolických přizpůsobení linií obývajících extrémní prostředí.

4.7. Buněčná struktura anaerobních heteroloboseí

4.7.1. Bičíkatý aparát u eukaryot a exkavát – struktura a terminologie

Bičíkatý aparát je jednou ze synapomorfií eukaryot (Baroin *et al*. 1988; Yubuki a Leander 2013) a v buňce se podílí na celé řadě důležitých procesů; určuje, mimo jiné, i její tvar. Hraje významnou roli zejména při pohybu buňky a přijímaní potravy. Tvoří jej bazální tělíska bičíků, jejich axonemy a celá řada různých cytoskeletárních útvarů, které v blízkosti bazálních tělísek přímo vznikají nebo jsou s nimi nějakým způsobem asociovány. Každé bazální tělísko bičíku je spojeno se specifickými cytoskeletárními elementy, v bičíkatém aparátu tak lze od sebe jasně odlišit jednotlivá bazální tělíska a homologizovat je s odpovídajícími bazálními tělísky jiných eukaryotických skupin (Moestrup 2000; Yubuki a Leander 2013; Yubuki *et al*. 2013).

Bazální tělíska v bičíkatém aparátu eukaryot jsou spolu vývojově spojena, neboť při tvorbě dceřiných aparátů stávající bazální tělíska zrají, mění svou pozici a kotví jiné cytoskeletární elementy. Je to dáno tím, že bazální tělíska potřebují víc než jeden buněčný cyklus ke svému zrání. Pouze nejstarší (zralé) bazální tělísko si uchovává svou identitu i v další generaci. Bazální tělíska transformovaná do vyššího stádia zralosti jsou v dceřiných aparátech doplněna nově vznikajícími nezralými bazálními tělísky. Tento jev se nazývá **ciliární transformace**, je svou povahou

semikonzervativní a byl popsán u řady eukaryotických linií (např. Beech *et al*. 1991; Brugerolle a Simpson 2004; Heimann *et al*. 1995; Melkonian *et al*. 1987; Nohýnková *et al*. 2006; Perasso *et al*. 1992; Yubuki a Leander 2012; Yubuki *et al*. 2013).

Mnohé z cytoskeletárních elementů asociovaných s bazálními tělísky jsou složeny z mikrotubulů. Obecně lze konstatovat, že se u eukaryot setkáváme s několika základními mikrotubulárními elementy, pro které byla zavedena jednotná terminologie: mikrotubulární kořeny R1 – R4, *singlet root* (dále jako singletový kořen) a dorzální vějíř mikrotubulů (Moestrup 2000; Yubuki *et al*. 2013). Posledně jmenovaná struktura přitom obvykle vzniká z mikrotubulárního organizačního centra umístěného na mikrotubulárním kořenu R3. U některých skupin se však setkáváme s natolik specifickými mikrotubulárními elementy, že je nelze jednoznačně homologizovat s konkrétním mikrotubulárním kořenem. Příkladem je konus archaméb (viz rozdílné interpretace v Yubuki a Leander 2013 a Walker *et al*. 2001).

Kromě mikrotubulárních útvarů lze v bičíkatém aparátu většiny eukaryotických skupin nalézt i různé nemikrotubulární fibrily. Může jít o jednoduché svazky vláken či pásy amorfní hmoty bez specifické vnitřní struktury. Často ale bývá struktura fibril složitější. Na řezech fibrilami pak lze s použitím transmisního elektronového mikroskopu rozeznat několik vrstev nebo pravidelně se opakujících modulů o různé elektrondenzitě (žíhání). Obecně rozšířenou nemikrotubulární fibrilou je vícevrstevná C fibrila, která je stabilně asociována s mikrotubulárním kořenem R1 (Yubuki a Leander 2013). U konkrétních eukaryotických linií lze ale nalézt i celou řadu dalších fibril. Bičíkatý aparát skupiny Excavata například obsahuje A fibrilu, B fibrilu a I fibrilu. Vzájemná homologie fibril mezi jednotlivými liniemi je často nejasná, a proto jsou u různých skupin označovány specifickými názvy (kosta trichomonád, rhizoplast heteroloboseí apod.) nebo jsou pojmenovávány velmi obecně – svazek mikrofilament, žíhaná fibrila. Složení a vlastnosti některých fibril byly detailně studovány, příkladem je rhizoplast druhu *Naegleria gruberi* (Chung *et al*. 2007; Larson a Dingle 1981), kosta trichomonád (např. Amos *et al*. 1979; Viscogliosi a Brugerolle 1993, 1994) nebo žíhaná fibrila *Chlamydomonas reinhardtii* (Lechtreck a Silflow 1997).

Homologizace jednotlivých komponent bičíkatého aparátu napříč různými skupinami eukaryot je poměrně složitá a vyžaduje detailní znalost prostorových a vývojových vztahů mezi jednotlivými částmi tohoto aparátu u studovaných linií. Je založena na třech základních principech: (1) pozice vůči jiným částem bičíkatého aparátu, (2) unikátní morfologické znaky typické pro určité elementy a (3) znalost vývojových vztahů mezi jednotlivými mikrotubulárními elementy během ciliární transformace. Při transformaci mladšího bazálního tělíska na zralé bazální tělísko se totiž z R3 stává R1 a z R4 vzniká R2 (Moestrup 2000; Yubuki *et al*. 2013).

Při studiu bičíkatých aparátů využíváme toho, že bazální tělíska bičíků jsou chirální struktury, které existují (pokud je známo) u všech eukaryot vždy ve stejné enantiomerní konfiguraci. Toho se

prakticky využívá při určování orientace buněčného řezu v elektronovém mikroskopu (Sleigh 1988). Protože se na konkrétní triplety určitých bazálních tělísek kotví konkrétní mikrotubulární kořeny, zakládá se tak asymetrie bičíkatého aparátu, a tím i polarita celé buňky. Polaritou buňky mám v tomto případě na mysli její strukturně či funkčně asymetrickou organizaci, která je zachovávána a předávána do dalších generací (definice dle Bornens 2008).

Polarita buněk zapříčiněná asymetrií bičíkatého aparátu je u řady eukaryot zřetelná již na první pohled. Příkladem je exkavátní bičíkovec rodu *Trimastix* (Excavata: Preaxostyla), u něhož se navenek tato polarita zřetelně projevuje asymetrií potravní rýhy (Simpson *et al*. 2000; Zhang *et al*., *in press*). Lze ji ale pozorovat také u zdánlivě symetrických buněk, například u zelené řasy druhu *Chlamydomonas reinhardtii*, kde polarita bičíkatého aparátu předurčuje polohu světločivné skvrny a kontraktilních vakuol (Holmes a Dutcher 1989; Marshall 2012).

V oblasti výzkumu struktury bičíkatého aparátu stále dochází k významným pokrokům, i když mechanismy udržující polaritu bičíkatého aparátu, nebo naopak indukující jeho přestavby jsou stále nedostatečně prozkoumány (viz Beisson a Jerka-Dziadosz 1999; Silflow a Lefebvre 2001). Ukazuje se, že architektura a složení bičíkatého aparátu jsou natolik konzervativní, že lze rekonstruovat plesiomorfní stav nejen u jednotlivých eukaryotických kmenů, nýbrž i u eukaryot jako celku. Yubuki a Leander (2013) například ukázali, že bičíkatý aparát posledního společného předka eukaryot (LECA) obsahoval dvojici bazálních tělísek, mikrotubulární kořeny R1 – R4, *singlet root*, dorzální vějíř mikrotubulů asociovaný s R3 a C fibrilu.

Potenciál studia struktury bičíkatého aparátu se ale neomezuje jen na akademickou debatu o vzhledu předků jednotlivých eukaryotických linií. Porovnávání struktury bičíkatého aparátu je možno využít i při odhalování příbuzenských vztahů, a to nejen na úrovni druhů, ale díky jeho konzervativní struktuře i k odhalování vztahů mezi vyššími taxony. Protože bičíkatý aparát hraje klíčovou roli při pohybu, přijímaní potravy a určuje základní tělní plán buňky, odhalení mechanismů vedoucích k jeho vnitřním přestavbám pravděpodobně přispěje k pochopení, proč jsou některé linie prvoků (bičíkovců) schopné vytvářet velké množství tělních plánů a jiné, ač ekologicky a druhově značně diverzifikované, si udržují jednotný tělní plán a poměrně stálou architekturu bičíkatého aparátu.

Ultrastruktura bičíkatého aparátu hrála důležitou roli při vytvoření superskupiny Excavata (Cavalier-Smith 2002; Simpson 2003; Simpson a Patterson 1999). Nutno podotknout, že monofylie této skupiny, zahrnující Discoba, Malawimonadea a Metamonada, nebyla přes veškeré snahy dodnes jednoznačně potvrzena metodami molekulární fylogenetiky. Fylogenomická studie Hampl *et al*. (2009) sice naznačovala monofylii exkavát včetně malawimonád, novější studie spíše ukazují, že Malawimonadea nejsou zbylým dvěma skupinám příbuzné (Derelle *et al*. 2015; Katz a Grant 2015; Zhao *et al*. 2012).

U volně žijících zástupců skupiny Excavata jsou přítomny všechny důležité elementy bičíkatého aparátu nacházené u ostatních eukaryot. Navíc obsahují i několik specifických elementů, nemikrotubulární fibrily A, B a I. Kvůli jeho složitosti a přítomnosti všech klíčových komponent považují Yubuki a Leander (2013) architekturu bičíkatého aparátu některých exkavát za stav připomínající předka všech dnes žijících skupin eukaryot. Pokud se prokáže, že *Malawimonas* a ostatní Excavata nejsou blízce příbuzní, bude to znamenat, že tato plesiomorfní architektura se zachovala u dvou různých linií eukaryot v téměř identické podobě a nemikrotubulární fibrily A, B a I byly přítomny už u jejich společného předka.

Za původní stav u exkavát je považována architektura bičíkatého aparátu založená na dvou bazálních tělíscích (Yubuki a Leander 2013). Zatímco mladší z těchto tělísek je namířeno dopředu (anteriorně) a je asociováno s mikrotubulárním kořenem R3 a s věncem superficiálních mikrotubulů, starší bazální tělísko zakládá posteriorně orientovaný bičík a je namířeno zhruba kolmo k bazálnímu tělísku mladšího bičíku; je asociováno s mikrotubulárním kořenem R1, R2, singletovým kořenem a s několika fibrilami (A, B, I, C). V případě druhu *Andalucia incarcerata* se dokonce zdá, že je na bazálním tělísku anteriorního bičíku zachován i mikrotubulární kořen R4 (Simpson a Patterson 2001).

Když se detailně podíváme na konfiguraci cytoskeletu kolem bazálního tělíska nejstaršího bičíku (posteriorního bičíku exkavátních taxonů), můžeme rozlišit dvě hlavní skupiny elementů, které jsme označili jako R2/I systém a R1/C systém (Pánek *et al.* 2014b). R1/C systém podporuje levou stranu potravní rýhy a je tvořen mikrotubulárním kořenem R1 podpořeným na konvexní straně C fibrilou. R2/I systém podporuje v závislosti na sledovaném taxonu pravou, střední nebo i levou část této rýhy. R2/I systém je složitější než R1/C, neboť kromě mikrotubulárního kořene R2 obsahuje i několik fibril (A, B, I). Navíc se R2 exkavát, stejně jako R2 jiných eukaryot, postupně rozestupuje do dvou větví. Vnější větev (oR2) je umístěna dál od bazálního tělíska, je asociována s B fibrilou a podporuje pravou stranu rýhy. Umístění vnitřní větve (iR2) je variabilnější, neboť může podkládat různé části potravní rýhy. Na konkávní straně je R2 podporováno I a B fibrilou, na konvexní pak A fibrilou (Yubuki a Leander 2013; Yubuki *et al*. 2013; Simpson 2003). Přinejmenším u druhu *Kipferlia bialata* (Excavata: Fornicata) je B fibrila asociována jak s R2, tak s R1 (Yubuki *et al*. 2013).

Ultrastrukturní studie publikované mezi roky 2000 a 2013, které využívaly Moestrupovu terminologii (Moestrup 2000; Simpson 2003), nesprávně identifikovaly mikrotubulární kořeny exkavát. Mikrotubulární kořen R1 tak byl u exkavát chybně označován jako R2 a naopak. Vedle toho se dodnes můžeme v literatuře setkat s alternativní terminologií - levý kořen (*left root*, syn. R1 *sensu* Yubuki *et al*. 2013) a pravý kořen (*right root*, syn. R2 *sensu* Yubuki *et al*. 2013) (viz Park a Simpson 2011; Simpson a Patterson 2001). Zásadní v tomto ohledu byla práce Yubuki *et al*. (2013), která detailně charakterizovala ciliární transformaci u druhu *Kipferlia bialata* a následnou reinterpretací struktury bičíkatého aparátu napravila chybu, které se dopustil Moestrup při interpretaci bičíkatého

aparátu skupiny Euglenozoa. U této skupiny totiž Moestrup obrátil označení mikrotubulárních kořenů v porovnání s ostatními eukaryoty. Na celá Excavata tuto obrácenou terminologii rozšířil Simpson (2003).

4.7.2. Bičíkatý aparát skupiny Heterolobosea a jeho evoluce

Většina bičíkovců heteroloboseí má v bičíkatém aparátu dva nebo čtyři bičíky. Zdá se ale, že původně byla Heterolobosea čtyřbičíkatá (Cavalier-Smith a Nikolaev 2008). R3 a R4 u heteroloboseí pravděpodobně zcela chybí a R1/C systém je znám pouze u podkmene Pharyngomonada (Park a Simpson 2011). Přestože u heteroloboseí chybí R3, mají vytvořeny dorzální mikrotubuly. Ty obvykle tvoří výrazný oblouk v přední části buňky.

Jak bylo popsáno výše, během ciliární transformace bazální tělísko 2. nejstaršího bičíku dozrává a jeho mikrotubulární kořeny R3 a R4 se stávají R1 a R2. U čtyřbičíkatých heteroloboseí ciliární transformace probíhá také, ale oproti klasickému scénáři je neobvyklá tím, že nejstarší a druhé nejstarší bazální tělísko nesou velmi podobné cytoskeletární útvary. Nejlépe je to vidět na příkladu druhu *Percolomonas sulcatus* (Brugerolle a Simpson 2004). Navíc jsou nejstarší a druhý nejstarší bičík umístěny v různých párech, každý ve dvojici s jedním bičíkem nulté generace. Nejstarší (druhá generace) a druhý nejstarší bičík (první generace) mateřské buňky slouží jako nejstarší bičík v dceřiných buňkách. Obě nejmladší tělíska (stejného stáří) se stávají druhým nejstarším bazálním tělískem v dceřiných buňkách, *de novo* se pak v každé z těchto buněk dotváří třetí a čtvrté bazální tělísko. Navenek se tedy čtyřbičíkatý aparát heteroloboseí jeví jako dvojice vývojově propojených dikinetid (Brugerolle a Simpson 2004). Ciliární transformace ale není nutnou podmínkou pro vznik bičíkatého aparátu za všech okolností. Améba druhu *Naegleria gruberi* neobsahuje žádná bazální tělíska a v určitých podmínkách se během pouhých dvou hodin transformuje v bičíkovce, aniž by se musela dělit (Dingle a Fulton 1966; Lee 2010; Walsh 2007). Tím pádem celý bičíkatý aparát těchto bičíkovců vzniká během jediné generace a v poměrně krátkém čase *de novo*. Toto pravděpodobně není výsada pouze *N. gruberi*. Ani u jiných heterolobózních améb nebyla dosud pozorována bazální tělíska, je tudíž možné, že se i u nich tvoří *de novo* při transformaci na bičíkovce.

My jsme studovali strukturu bičíkatého aparátu u heteroloboseí druhů *Creneis carolina* (Pánek *et al.* 2014b), *Monopylocystis visvesvarai* (Pánek *et al.* 2012), *Pseudoharpagon tertius* a *P. pertyi* (Pánek *et al*. 2014a). U dvou ze jmenovaných druhů, *C. carolina* a *P. pertyi*, jsme provedli detailní ultrastrukturní studii. Definovali jsme synapomorfii bičíkatého aparátu čeledi Psalteriomonadidae (Pánek *et al.* 2012) a zavedli jsme terminologii mikrotubulárních kořenů dle Yubuki *et al*. (2013) u heteroloboseí (Pánek *et al.* 2014a). Zatímco struktura bičíkatého aparátu u čeledi Psalteriomonadidae dobře odpovídá tomu, co je známo u ostatních heteroloboseí, *Creneis*

carolina je v tomto ohledu unikátní nejen na úrovni kmene Heterolobosea, ale pravděpodobně i na úrovni eukaryot jako takových.

Ačkoliv bičíkovci heteroloboseí byli původně zřejmě čtyřbičíkatí, řada druhů má bičíkatý aparát jen se dvěma bičíky, například *Pleurostomum flabellatum* nebo *Heteramoeba clara* (Park *et al*. 2007; Droop 1962). U některých linií byl zaznamenán variabilní počet bičíků v bičíkatém aparátu. Například u druhu *Naegleria gruberi* je většina bičíkovců dvoubičíkatá, ačkoliv za normálních podmínek lze nalézt i jedno-, tří- a čtyřbičíkaté buňky (Dingle a Fulton 1966). Je-li transformace améby na bičíkovce indukována za zvýšené teploty (38 ^{o}C), tvoří se bičíkovci až s 18 bičíky. Přinejmenším polovina buněk nese za těchto okolností 5 a více bičíků. Po opětovné přeměně takových anomálních bičíkovců na améby a zpět se za normálních podmínek tvoří opět převážně dvoubičíkaté buňky (Dingle 1970). Podobně je počet bičíků variabilní i u bičíkovců převážně dvoubičíkatých druhů *Tetramitus jugosus* a *Oramoeba fumarolia* (Darbyshire *et al*. 1976; De Jonckheere *et al*. 2011), detailní data o jejich uspořádání v mastigontech ale chybí.

Bičíkovci čeledi Psalteriomonadidae jsou převážně čtyřbičíkatí, existují však dvě výjimky. První je druh *Psalteriomonas lanterna*, jehož buňky mají čtyři identické a symetricky rozmístěné bičíkaté aparáty, každý se čtyřmi bičíky (Broers *et al*. 1990). Tyto bičíkovci mají i čtyři jádra a čtveřici exkavátních rýh. Druhý případ jsme popsali u druhu *Pseudoharpagon longus* (Pánek *et al*. 2014a). Na buňkách obarvených protargolem jsme sledovali nejen počet bičíků, ale i počet bičíkatých aparátů, jader a exkavátních rýh. Tento druh má většinou pět bičíků v mastigontu, což je dosti neobvyklé. Bičíkovci, kteří mají mastigont s více než dvěma bičíky mají totiž nejen u ostatních heteroloboseí, ale i eukaryot většinou sudý počet bičíků. Snad to souvisí se semikonzervativním charakterem ciliární transformace. Na základě dostupných dat z preparátů barvených protargolem se zdá, že by uvnitř rodu *Pseudoharpagon* mohla existovat variabilita i pokud jde o načasování rozdělení bičíkatého aparátu vzhledem k dělení jádra (srv. obr. 9M v Pánek *et al*. 2012 a obr. 5K v Pánek *et al*. 2014a).

Výše zmíněné příklady ilustrují, že Heterolobosea jsou skupinou eukaryot, která relativně velmi snadno mění architekturu svého bičíkatého aparátu a dokáže zřejmě obejít i proces ciliární transformace. Nejvýraznější doposud známou ukázku přeměny bičíkatého aparátu heteroloboseí jsme ale popsali u druhu *Creneis carolina* (Pánek *et al*. 2014b), který představuje jedinou známou linii heteroloboseí, jež má v mastigontu jeden bičík. Jde také o jediného známého příslušníka tohoto kmene, který stabilně nese bičíky v améboidním stádiu. My jsme studovali strukturu bičíkatého aparátu tohoto jednobičíkatého améboidního stádia (hlavní trofické stádium). Tento bičík se nám podařilo homologizovat s nejstarším bičíkem ostatních eukaryot. Jeho bazální tělísko nese čtyři nemikrotubulární elementy a dva mikrotubulární kořeny, R1 a R2. Identitu R2, respektive R2/I systému jsme potvrdili díky několika specifickým znakům, které sdílí s ostatními zástupci kmene Heterolobosea i jinými eukaryoty. Z našich dat ale zároveň plyne, že R2/I systém, nebo dokonce celý

bičíkatý aparát *C. carolina*, prošel reverzí chirality oproti tomu, co známe u jiných linií. Alternativní hypotézou, kterou nemůžeme vyloučit, avšak považujeme ji za mnohem méně pravděpodobnou, je možnost, že reverzí chirality prošel bičík a jeho bazální tělísko. Ať už platí jedna nebo druhá hypotéza, jde o dosud zřejmě bezprecedentní změnu ve vývoji bičíkatého aparátu eukaryot.

Reverze chirality vyplývá z detailního studia R2/I systému, který lze u *Creneis carolina* na základě několika unikátních charakteristik jasně identifikovat – mikrotubulární kořen R2 je asociován s fibrilou připomínající svou mřížovanou stukturou I fibrilu ostatních heterolobosei; na R2 se připojuje žíhaná fibrila připomínající rhizoplast; kořen R2 je rozdělen na dvě části a na cytoplasmatické straně R2 je část mikrotubulů podložena vzájemně propojenými paprsky. Reverzi chirality ovšem naznačují poziční charakteristiky R2/I systému - R2 u *C. carolina* vzniká na levé straně bazálního tělíska (nikoliv na pravé); rhizoplast vzniká na pravé straně R2 (nikoliv na levé); je to vnitřní část R2 (R2a), která vede dolů na pravé straně buňky, kdežto u zástupců čeledi Psalteriomonadidae a u rodu *Pleurostomum* tuto stranu buňky podporuje vnější část R2. R2 identifikovaný u *C. carolina* tak velmi připomíná R2 ostatních heterolobosei, až na to, že je jeho zrcadlovým obrazem.

4.7.3. Mitochondrie a její morfologie u anaerobních heterolobosei

Mitochondrie je jednou ze základních buněčných organel eukaryot a byla přítomna již u předka všech dnes žijících eukaryotických linií (např. Dolezal *et al*. 2006; Hrdy *et al*. 2004; Embley a Hirt 1998; Roger 1999; Roger *et al*. 1998). Vznikla endosymbiózou z α-proteobakterie (Gray *et al*. 1999, 2001; Margulis 1970) a během evoluce eukaryot se rozrůznila do celé řady variant, které se liší svou morfologií i množstvím metabolických drah, jež v nich probíhají (viz Maquire a Richards 2014; Müller *et al*. 2012;). Ze všech známých biosyntetických drah lokalizovaných v mitochondrii je zřejmě nejméně postradatelnou tvorba Fe-S klastrů. Tato dráha má esenciální roli při tvorbě FeS proteinů v celé buňce (Lill a Kispal 2000; Lill *et al*. 1999).

Při pohledu transmisním elektronovým mikroskopem můžeme v mitochondrii rozlišit čtyři základní části: vnější membránu, vnitřní membránu, mezimembránový prostor a mitochondriální matrix. U klasických mitochondrií je vnitřní membrána ještě rozdělena do dvou funkčně odlišných oddílů, které označujeme jako vnitřní obalová membrána (dále IBM – „inner boundary membrane“) a mitochondriální kristy. IBM přiléhá poměrně těsně k vnější mitochondriální membráně, kdežto kristy jsou invaginace vnitřní membrány do mitochondriální matrix. Na membráně krist jsou nahromaděné komplexy dýchacího řetězce a F_1-F_O ATP syntáza. IBM zase vykazuje silnou koncentraci translokáz důležitých pro přenášení proteinů (Wurm a Jakobs 2006). Vysoká koncentrace enzymů důležitých pro oxidativní fosforylaci na mitochondriálních kristách dobře vysvětluje, proč řada prvoků, kteří žijí

v anoxickém nebo mikrooxickém prostředí a nevyužívají dýchací řetězec při tvorbě ATP, mitochondriální kristy zcela ztratila.

V minulosti se předpokládalo, že se některé linie anaerobních eukaryot v evoluci oddělily ještě před vznikem klasické mitochondrie. Tyto linie se sdružovaly, jak již bylo uvedeno výše, do skupiny Archezoa (Cavalier-Smith 1987). Později se však podařilo prokázat stejný původ klasických mitochondrií, hydrogenosomů a mitosomů a podařilo se objevit MROs v organismech, kde předtím nebyly známy (např. Embley a Hirt 1998; Roger 1999). Jak se rozšiřovaly znalosti o fungování mitochondriálních derivátů (MROs) různých fakultativně či obligátně anaerobních linií a odhalovala se různorodost jejich enzymatické výbavy, vznikl systém rozdělující mitochondrie a jejich deriváty do pěti základních typů podle toho, zda jsou schopny aerobní produkce ATP (klasické mitochondrie), anaerobní produkce ATP (anaerobní mitochondrie, hydrogenosomy, vodík produkující mitochondrie), nebo nemají vůbec schopnost produkovat ATP (mitosomy) (Müller *et al*. 2012). Z těchto pěti typů pouze mitosomy a hydrogenosomy neobsahují kristy.

Dnes je zřejmé, že MROs nelze takto jednoduše roztřídit do několika distinktních skupin, neboť mezi nimi existují přechody (Maquire a Richards 2014; Stairs *et al*. 2014). V každém případě ale platí, že chybí-li v mitochondriích určitého organismu kristy, jedná se o anaerobní derivát mitochondrie s pozměněnou nebo zcela redukovanou schopností produkce ATP v důsledku života bez kyslíku. Jinak řečeno, akristátní mitochondriální deriváty se vyznačují redukcí dýchacího řetězce a absencí chemiosmotické produkce ATP.

Nedávno se zjistilo, že tvorba mitochondriálních krist je podmíněna multiproteinovým komplexem MICOS (viz Pfanner *et al*. 2014), jehož klíčová součást (Mic60) pochází z původního α-proteobakteriálního předka mitochondrie a přinejmenším geny pro další dvě důležité části tohoto komplexu (Mic10 a Mic19) byly přítomny u společného předka dnešních eukaryot. Absence těchto proteinů MICOS komplexu koreluje s absencí mitochondriálních krist u eukaryot (Muñoz-Gómez *et al*. 2015), což podporuje představu, že u všech anaerobů s akristátními mitochondriemi došlo až k druhotné ztrátě krist a s nimi svázaných metabolických drah, tedy že absence krist je apomorfní stav.

Většina heteroloboseí má klasické mitochondrie s diskoidálními kristami (viz např. Page a Blanton 1985), zatímco u všech tří druhů rodu *Psalteriomonas* (Broers *et al*. 1990, 1993; de Graaf *et al*. 2009; Pánek *et al*. 2012), u druhu *Monopylocystis visvesvarai* (O´Kelly *et al*. 2003; Pánek *et al*. 2012), *Sawyeria marylandensis* (Barberà *et al*. 2011), *Harpagon descissus* (Brugerolle a Simpson 2004) (vše Psalteriomonadidae) a *Creneis carolina* (Pánek *et al*. 2014b) (Creneidae) byly pozorovány pouze akristátní mitochondriální deriváty. Přechodový stupeň mezi klasickými a akristátními mitochondriemi by mohl představovat druh *Pseudoharpagon pertyi* (Psalteriomonadidae), v jehož

mitochondriálních derivátech byly pozorovány invaginace vnitřní mitochondriální membrány připomínající individuální diskoidální kristy (Pánek *et al.* 2014a).

Zdálo by se, že morfologie akristátních mitochondriálních derivátů eukaryot musí být u všech anaerobních linií téměř identická, neboť to jsou jen váčky obalené dvěma membránami. Právě u anaerobních heterolobosei toto ale neplatí. Rod *Psalteriomonas* je zajímavý tím, že jeho buňky obsahují mitochondriální deriváty dvou různých morfotypů. První typ tvoří mohutný shluk hydrogenosomů asociovaný s methanogenními archebakteriemi (hydrogenosomální komplex) a druhý typ představují individuální hydrogenosomy jednotlivě rozmístěné v buňce. Zatímco hydrogenosomální komplex byl pozorován u všech tří druhů tohoto rodu v bičíkatém (Broers *et al*. 1990, 1993; de Graaf *et al*. 2009) i amébovitém stádiu (Broers *et al*. 1993; Pánek *et al.* 2012), individuální organely byly identifikovány pouze u *P*. *lanterna* a *P*. *magna* (Broers *et al*. 1990; Pánek *et al.* 2012). Umístění hydrogenosomálního komplexu je v rámci daného druhu a stádia stabilní a jeho přítomnost byla prokázána i v buňkách pěstovaných dlouhou dobu bez methanogenních symbiontů (Broers *et al*. 1993; de Graaf *et al*. 2009). U druhu *Psalteriomonas lanterna* jsou individuální MROs, na rozdíl od těch nahloučených v hydrogenosomálním komplexu, obaleny hrubým endoplasmatickým retikulem.

Druh *Sawyeria marylandensis* představuje další morfologicky unikátní stav. Bylo prokázáno, že tyto buňky obsahují hydrogenosomy, které mají pohárkovitý tvar (Barberà *et al*. 2011).

Morfologie MROs druhu *Monopylocystis visvesvarai* je naopak velmi podobná akristátním MROs jiných anaerobních eukaryot (jde o kulovité akristátní MROs). Ty jsou, podobně jako klasické mitochondrie mnohých heterolobosei, často umístěny v blízkosti cisteren drsného endoplasmatického retikula (Pánek *et al.* 2012). Podobně mají kulovitý tvar také MROs druhu *Creneis carolina*, avšak nejsou asociovány s drsným endoplasmatickým retikulem (Pánek *et al.* 2014b).

V kontextu funkce mitochondriálních krist je velmi zajímavý zmíněný objev zbytků krist v MROs druhu *Pseudoharpagon pertyi* (Pánek *et al.* 2014a). Domnívám se, že tyto organely by mohly představovat přechod mezi klasickými mitochondriemi heterolobosei a hydrogenosomy příslušníků čeledi Psalteriomonadidae.

Kmen Heterolobosea je vhodnou modelovou skupinou výzkumu vzniku anaerobního způsobu života u eukaryot, neboť obsahuje několik obligátně (Creneidae, Psalteriomonadidae) a pravděpodobně i fakultativně anaerobních linií (např. *Percolomonas sulcatus, Naegleria gruberi* - viz výše). Za pozornost stojí i přítomnost zbytků mitochondriálních krist u *Pseudoharpagon pertyi*, který tak pravděpodobně představuje jiný typ anaerobních MROs než u ostatních zástupců Psalteriomonadidae. Vzhledem k množství linií schopných alespoň dočasně prosperovat v kyslíkem chudém prostředí se navíc nabízí hypotéza, že už předek heterolobosei byl vybaven některými enzymy anaerobního metabolismu, což usnadnilo vznik anaerobních linií v pozdější evoluci tohoto

kmene. Rozhodli jsme se proto z několika druhů anaerobních i aerobních heterolobosei získat EST data, z druhu *Neovahlkampfia damariscottae* jsme získali navíc i draft genomu (přehled viz **tabulka 2**). Tato data v současnosti analyzuji. *In silico* rekonstrukci metabolismu však komplikuje fakt, že mitochondriální targetovací sekvence u heterolobosei jsou nekanonické, a proto obtížně predikovatelné s použitím softwarových nástrojů (Barberà *et al*. 2010; Pánek *et al*. nepubl.). Navíc bylo nedávno zjištěno, že některé potenciální mitochondriální proteiny druhu *Naegleria gruberi* naopak nesou N-terminální peptid, v němž je *in silico* poměrně jasně rozpoznávaný mitochondriální targetovací signál, ačkoliv jsou tyto proteiny ve skutečnosti lokalizovány v cytoplasmě (Tsaousis *et al*. 2014).

4.8. Budoucí směry výzkumu heterolobosei, využití transkriptomických dat

Genomická studie druhu *Naegleria gruberi* (Fritz-Laylin *et al*. 2010) ukázala, že tento organismus disponuje jednak geny klasického aerobního metabolismu, včetně kompletního setu enzymů dýchacího řetězce, jednak obsahuje i geny několika enzymů typických pro anaerobní metabolismus. Zejména se jedná o [FeFe]-hydrogenázu (HydA), všechny tři její maturázy (HydE, HydF, HydG) a některé Ppi-dependentní enzymy glykolýzy (Ppi-dependentní fosfofruktokináza a puruvát-fosfát dikináza). [FeFe]-hydrogenáza je enzym citlivý na kyslík a typický pro anaerobní a mikroaerofilní eukaryota. Přes ferrodoxin odebírá elektrony uvolňované při anaerobní přeměně pyruvátu na acetyl-CoA a CO_2. Tyto elektrony pak [FeFe]-hydrogenáza přenáší na protony za vzniku molekulárního vodíku (Mulder *et al*. 2011).

In silico predikce identifikovaly v sekvenci genu [FeFe]-hydrogenázy a maturáz druhu *Naegleria gruberi* N-terminální sekvence adresující daný protein do mitochondrie. To naznačovalo, že mitochondrie *N*. *gruberi* je schopna přepnout při nedostatku kyslíku na anaerobní metabolismus (Fritz-Laylin *et al*. 2010). Tsauosis *et al*. (2014) později experimentálně ukázali, že buňky *N*. *gruberi* skutečně produkují vodík a [FeFe]-hydrogenáza je v nich metabolicky aktivní. Překvapivě však s využitím imunolokalizace demonstrovali, že [FeFe]-hydrogenáza a minimálně jedna její maturáza (HydE) jsou v buňkách *N*. *gruberi* lokalizovány v cytoplasmě, nikoliv v mitochondrii. Protože je jen obtížně představitelné, že by ostatní maturázy měly jinou buněčnou lokalizaci než HydE a [FeFe]-hydrogenáza, předpokládá se, že i ony jsou umístěny v cytoplasmě. *N*. *gruberi* je jediným známým eukaryotem s touto lokalizací [FeFe]-hydrogenázy a jejích maturáz. Role [FeFe]-hydrogenázy v metabolismu *N*. *gruberi* zůstává neobjasněna, neboť se neví, s jakou dráhou je její činnost spojena.

Evoluční historie anaerobního energetického metabolismu u eukaryot je stále poměrně nejasná, existují dva základní scénáře. První tvrdí, že přítomnost klíčových anaerobních enzymů u různých skupin reflektuje anaerobní charakter předka eukaryot (Martin 2011; Martin a Müller 1998),

kdežto druhý scénář vychází z představy vícenásobného horizontálního genového přenosu. Druhý scénář je pravděpodobný například v případě [FeFe]-hydrogenázy, která byla do genomu eukaryot horizontálně přenesena minimálně dvakrát nezávisle na sobě (Hug *et al*. 2010; Nývltová *et al*. 2015). Naopak, jednotný původ by mohly u eukaryot mít maturázy [FeFe]-hydrogenázy (Hug *et al*. 2010; Jerlström-Hultqvist *et al*. 2013) a pyruvát:ferrodoxin oxidoreduktáza (PFO) (Leger *et al*. 2012; Nývltová *et al*. 2015; Stairs *et al*. 2014). Dosavadní fylogenetické analýzy však nejsou schopny fylogenezi těchto genů rozřešit s dostatečně vysokou statistickou podporou, to se snad v budoucnu zlepší se vzrůstajícím počtem sekvencí a se zdokonalováním modelů používaných ve fylogenetických analýzách.

Z obligátně anaerobních heterolobosei byl doposud studován mitochondriální metabolismus dvou blízce příbuzných druhů čeledi Psalteriomonadidae, konkrétně *Psalteriomonas lanterna* (de Graaf *et al*. 2009) a *Sawyeria marylandensis* (Barberà *et al*. 2011). V obou případech se jednalo o *in silico* analýzu omezeného množství EST dat. Předpokládá se, že oba organismy mají hydrogenosomy obsahující [FeFe]-hydrogenázu a její maturázy. Pyruvát dehydrogenáza (PDC), přeměňující u aerobních eukaryot pyruvát na acetyl-CoA, byla u psalteriomonadidů nahrazena PFO. Zachovány jsou u *S. marylandensis* i další metabolické dráhy, což naznačuje relativně komplexní anaerobní mitochondriální metabolismus MROs včetně metabolismu aminokyselin a mastných kyselin. Vizuální porovnání sekvencí získaných z druhu *S. marylandensis* s bakteriálními homology ukázalo N-terminální adresovací peptidy. Software běžně používaný k odhalování mitochondriálních adresovacích sekvencí ale dokázal potvrdit mitochondriální lokalizaci pouze v několika málo případech. N-terminální mitochondriální adresovací sekvence u studovaných zástupců Psalteriomonadidae jsou nekanonické, chybí v nich například kodóny pro arginin (Barberà *et al*. 2011).

Pokud se týká evoluce anaerobiózy u heterolobosei, je třeba v budoucnu odpovědět na několik základních otázek: (1) Byly geny anaerobního metabolismu nalezené i u *Naegleria gruberi* získány nezávisle na anaerobních liniích, anebo od společného předka? (2) Jak se liší anaerobní metabolismus zástupců čeledí Creneidae a Psalteriomonadidae? (3) Které enzymy či celé metabolické dráhy byly získány u těchto skupin nezávisle pomocí horizontálního genového transferu? (4) Která linie prokaryot či eukaryot byla donorem těchto genů? (5) Jak se liší mitochondriální metabolismus mezi jednotlivými liniemi skupiny Psalteriomonadidae a má *Pseudoharpagon* ještě zachovány dráhy vázané na vnitřní mitochondriální membránu?

Na tyto základní otázky pak lze navázat řadu dalších, jejichž zodpovězení pouze na základě *in silico* analýz je u heterolobosei bohužel v současnosti nemožné. Například je třeba zodpovědět otázku lokalizace klíčových anaerobních enzymů. Teprve pak se lze ptát, kde v buňce byla původně lokalizována [FeFe]-hydrogenáza a další enzymy anaerobního energetického metabolismu. Definitivní

rozřešení otázky lokalizace jednotlivých proteinů si však žádá použití jiných metod (imunolokalizace, analýza proteomu mitochondriálních derivátů).

V rámci probíhajícího projektu zaměřeného na *in silico* predikci anaerobního metabolismu u heterolobosei bylo mým úkolem napěstovat kultury vybraných druhů, extrahovat totální RNA a analyzovat získaná data. Vlastní analýza probíhá ve spolupráci s Dr. Courtney Stairs a Mgr. Eliškou Zadrobílkovou. Založili jsme ji na identifikaci zájmových enzymů pomocí BLAST (protein BLAST proti nr databázi v GenBank). Následně jsme u vybraných enzymů hledali proteinové domény či typické motivy, na základě kterých lze dané enzymy identifikovat nebo rozhodnout o jejich funkci. Pomocí programů MITOPROT, PSORT II a TargetP 1.1. jsme hledali mitochondriální adresovací sekvence. Ve vybraných případech jsme provedli i fylogenetické analýzy. Analýza dat stále probíhá, přesto bych zde chtěl zmínit několik dílčích výsledků.

Predikovaný mitochondriální metabolismus druhu *Harpagon schusteri* se zdá být velmi podobný druhu *Sawyeria marylandensis* (oba Psalteriomonadidae), ačkoliv naše data z *H*. *schusteri* jsou kompletnější než publikovaná data *S*. *marylandensis*. Velkým problémem ovšem je, že pouze malá frakce potenciálních mitochondriálních proteinů obsahuje N-terminální mitochondriální adresovací peptidy. Zatímco v datech ze *S*. *marylandensis* byl jako jediný enzym přeměňující pyruvát na acetyl-CoA identifikován PFO, nám se podařilo u *H*. *schusteri* kromě PFO identifikovat v EST datech i všechny tři podjednotky pyruvát dehydrogenázy (PDC), z nichž jedna podjednotka dokonce nese N-terminální extenzi, která je pravděpodobně mitochondriální adresovací peptid. Žádná z kopií PFO však není *in silico* predikována do mitochondrie. Bez ohledu na lokalizaci PFO je *Harpagon* jedním z mála eukaryot, u nichž koexistuje PDC a PFO. Podobná situace je známa ještě u rodů *Blastocystis*, *Perkinsus* a *Acanthamoeba* (viz Tsaousis *et al*. 2012).

Psalteriomonadidae a Creneidae zřejmě ztratily mnoho komponent aerobního metabolismu (více viz **obrázek S2 v příloze**), ačkoliv je třeba upozornit, že naše znalosti vychází z EST dat, nikoliv z dat genomických. Například z enzymů dýchacího řetězce jsme v obou případech identifikovali pouze dvě podjednotky komplexu I, konkrétně NuoE a NuoF. Tyto podjednotky jsou zachovány třeba i v hydrogenosomech *Trichomonas vaginalis* (Excavata: Parabasalia), kde reoxidují NADH vznikající při přeměně malátu na pyruvát (viz Müller *et al*. 2012). U zástupců Creneidae a Psalteriomonadidae chybí i řada enzymů Krebsova cyklu (**viz obrázek S3 v příloze**). Bohužel zatím nemáme k dispozici EST data z druhu *Pseudoharpagon pertyi*, který by mohl mít tyto dvě dráhy zachovány v kompletnější podobě, protože má zřejmě ještě mitochondriální kristy.

Podobně nás zajímal výskyt různých enzymů glykolýzy (**viz obrázek S3 v příloze**). Naše výsledky ukazují, že předek dnešních heterolobosei (přinejmenším celého podkmene Tetramitia) zřejmě disponoval dvěma Ppi-dependentními enzymy glykolýzy. Podařilo se nám identifikovat PPDK, dříve nalezené v genomu *Naegleria gruberi*, v našich datech z *Creneis carolina* a *Harpagon schusteri*.

Následná fylogenetická analýza s bootstrapovou podporou 98 potvrdila, že PPDK získané z heteroloboseí tvoří monofylum, které je navíc sesterské k PPDK euglenozoí, byť s nízkou bootstrapovou podporou (**obrázek S4 v příloze**). To naznačuje, že gen pro PPDK byl získán nejen před divergencí skupiny Tetramitia, ale snad i celé skupiny Discicristata (Euglenozoa + Heterolobosea). Ortholog Ppi-dependentní fosfofruktokinázy identifikované v genomu *N. gruberi* (Fritz-Laylin *et al.* 2010) se nám podařilo naléz ještě ve větším počtu heteroloboseí, konkrétně u anaerobních druhů *Harpagon schusteri* a *Creneis carolina* a u aerobů *Heteramoeba clara*, *Neovahlkampfia damariscottae* a *Naegleria fowleri*. Nejvíce nás ale překvapil objev [FeFe]-hydrogenázy a všech jejích maturáz u aerobního druhu *N. damariscottae*, který se v naší fylogenomické analýze větví sestersky k ostatním zástupcům podkmene Tetramitia. Bylo potvrzeno, že [FeFe]-hydrogenáza je nejen přítomna v genomu druhu *Neovahlkampfia damariscottae*, ale je zde se svými maturázami i aktivně transkribována (nalezena v EST datech). Zatímco *Naegleria gruberi* a *Neovahlkampfia damariscottae* mají jen jednu kopii [FeFe]-hydrogenázy, Psalteriomonadidae mají nejméně dvě její kopie a Creneidae dokonce tři (viz **obr. S5A**). Dvě z nich jsou pravděpodobně stejného původu jako [FeFe]-hydrogenáza ostatních heteroloboseí, fylogenetická pozice třetí kopie nebyla fylogenetickými analýzami rozřešena. Je proto možné, že tato hydrogenáza byla získána pomocí LGT z jiného zdroje (viz **obr. S6**). Na základě těchto dat je dále velmi pravděpodobné, že [FeFe]-hydrogenázu a její maturázy měl již společný předek heteroloboseí, respektive podkmene Tetramitia.

V genomu *N. damariscottae* tvoří geny maturáz a [FeFe]-hydrogenázy metabolický genový klastr, přičemž jsou umístěny na úseku o délce přibližně 6,7 kbps (viz **obr. S5B**). Naznačuje to, že byly získány jediným horizontálním genovým přenosem z neznámého dárce. Ačkoliv [FeFe]-hydrogenáz mají anaerobní linie více kopií, maturázy se nacházejí vždy v jediné kopii a mají u heteroloboseí s vysokou pravděpodobností jednotný původ (viz **obr. S7**)

Není dosud jasné, z jaké konkrétní reakce [FeFe]-hydrogenáza odebírá elektrony v případě metabolismu *Neovahlkampfia damariscottae* a *Naegleria gruberi*, v jejichž genomech nebyly odhaleny žádné enzymy přeměňující pyruvát anaerobní cestou na acetyl-CoA. Naproti tomu u Psalteriomonadidae a u *Creneis carolina* je situace poněkud jasnější. V EST datech z těchto obligátně anaerobních linií jsme identifikovali eukaryotické PFO. *C. carolina* má dokonce několik PFO, které ve fylogenetických analýzách klastrují s ostatními eukaryotickými PFO, ale netvoří spolu monofyletickou skupinu (rozpadají se do dvou linií). Přinejmenším dvě PFO z *C. carolina* se větví sestersky k diplomonádám (Excavata: Fonicata), a to se střední statistickou podporou (s bootstrapovou podporou 68, viz **obr. S8**).

V této souvislosti je zajímavé, že také acetyl-CoA syntetáza (ACS) nalezená u *Creneis carolina* se větví sestersky k diplomonádám, tentokrát však s absolutní statistickou podporou (viz **obr. S9**). Naznačuje to, že mezi Creneidae a diplomonádami došlo k výraznému horizontálnímu genovému

přenosu enzymů důležitých pro anaerobní energetický metabolismus. U diplomonád slouží ACS k produkci ATP přemenou acetyl-CoA na acetát. Tato reakce tak bezprostředně následuje po přeměně pyruvátu na acetyl-CoA činností enzymu PFO. V případě druhu *Giardia intestinalis* probíhá zmíněná reakce v cytoplasmě, u druhu *Spironucleus salmonicida* pak zřejmě v hydrogenosomech (Jerlström-Hultqvist *et al*. 2013). Nývltová *et al*. (2015) prokázali, že ACS u obligátně anaerobní archaméby druhu *Mastigamoeba balamuthi* funguje jak v cytoplasmě, tak v hydrogenosomech.

ACS proteiny eukaryota získala nejméně čtyřmi nezávislými horizontálními genovými transfery: (i) ze skupiny Archaea do rodu *Mastigamoeba*; (ii) ze skupiny Firmicutes do diplomonád; (iii) z neznámého donora do rodu *Entamoeba*; (iv) z neznámého donora do dalších eukaryot, například i do rodu *Naegleria* (Nývltová *et al*. 2015). *Creneis* má acetyl-CoA syntetázu, která ve fylogenetických analýzách tvoří monofylum s ACS diplomonád a obsahuje N-terminální extenzi. Žádný jiný eukaryot tento homolog nemá a ostatní heterolobosea mají ACS ze stejného zdroje jako *Naegleria*. To naznačuje, že ACS u *C. carolina* bylo získáno nezávisle na ostatních heteroloboseích.

Na závěr této kapitoly lze shrnout, že předek podkmene Tetramitia pravděpodobně disponoval [FeFe]-hydrogenázou (hydA) a všemi třemi jejími maturázami (HydE, HydF, HydG). Později v evoluci obligátně anaerobních linií došlo k duplikaci HydA, a to zřejmě nezávisle u rodu *Harpagon* a *Sawyeria* (oba Psalteriomonadidae) a u *Creneis* (Creneidae). Již předek skupiny Tetramitia také disponoval dvěma Ppi-dependentními enzymy glykolýzy – pyruvát fosfát dikinázou (PPDK) a Ppi-dependentní fosfofruktokinázou (Ppi-PFK). Alespoň PPDK byla pravděpodobně získána ještě před oddělením linií vedoucích k heteroloboseím na jedné straně a ke skupině Euglenozoa na straně druhé. Obecně se má za to, že glykolýza s využitím těchto dvou enzymů je schopna produkovat 2,5krát více ATP než klasická Embden-Meyerhof-Parnasova glykolýza fungující u většiny eukaryot. Zvýšení efektivity glykolýzy je důležité zejména v případě, že nelze zapojit pro tvorbu ATP Krebsův cyklus a oxidativní fosforylaci, které jsou u většiny eukaryot zodpovědné za produkci až 95 % ATP v buňce (viz např. Slamovitz a Keeling 2006). Lze tedy shrnout, že Heterolobosea nebo přinejmenším Tetramitia disponovala již od počátku některými enzymy umožňujícími efektivnější energetický metabolismus v anoxickém a mikrooxickém prostředí (Ppi-PFK a PPDK) a enzymy, které jsou důležitou součástí anaerobního metabolismu pyruvátu u anaerobních prvoků (HydA, HydE, HydF, HydG). Další klíčové enzymy energetického metabolismu obligátně anaerobních heteroloboseí pak byly získány horizontálním genovým přenosem.

Dobrým příkladem takového genového přenosu je acetyl-CoA syntetáza (ACS), která má u rodů *Creneis* a *Naegleria* jasně odlišný evoluční původ. Podobně lze uvažovat i o genu pro pyruvát:ferrodoxin oxidoreduktázu (PFO) u *Creneis* a Psalteriomonadidae. Nelze opominout ani fakt, že předpokládaným zdrojem obou těchto horizontálně přenesených genů u *Creneis* jsou zástupci linie Fornicata.

5. Odborné publikace zahrnuté do této práce

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Pánek, T., Simpson, A.G.B., Hampl, V., Čepička, I. (2014b). *Creneis carolina* gen. et sp. nov. (Heterolobosea), a novel marine anaerobic protist with strikingly derived morphology and life cycle. Protist 165: 542-567.

Pánek, T., Táborský, P., Pachiadaki, M.P., Hroudová, M., Vlček, Č., Edgcomb, V.P., Čepička I. Combined culture-based and culture-independent approaches provide insights into diversity of jakobids, extremely plesiomorphic eukaryotic lineage. Rukopis je připraven k zaslání do tisku.

5.1. Pánek *et al.* 2012

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ORIGINAL PAPER

Diversity, Evolution and Molecular Systematics of the Psalteriomonadidae, the Main Lineage of Anaerobic/Microaerophilic Heteroloboseans (Excavata: Discoba)

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We isolated and cultivated 31 strains of free-living heterolobosean flagellates and amoebae from freshwater, brackish, and marine sediments with low concentrations of oxygen. Phylogenetic analysis of small subunit (SSU) rDNA showed that the strains constitute a single clade, the Psalteriomonadidae. According to combined light-microscopic morphology plus molecular phylogeny, our isolates belong to seven species and five genera, from which three species and two genera are new. In addition, previously described anaerobic species *Percolomonas descissus* and *Vahlkampfia anaerobica* are transferred to the Psalteriomonadidae. We identified a flagellate stage of *Monopylocystis visvesvarai* which was reported to produce only amoebae. Two environmental sequences previously obtained from acidic environments belong to the Psalteriomonadidae as well, suggesting a broad ecological importance of the Psalteriomonadidae. The ultrastructure of two psalteriomonadid species was also studied. Unifying features of the Psalteriomonadidae are acristate mitochondrial derivates, flagellates with a ventral groove and four flagella, and a harp-like structure in the mastigont. A new overall classification of the Psalteriomonadidae is proposed. Our data show that the Psalteriomonadidae are much more diverse than previously thought and constitute the main anaerobic lineage within the Heterolobosea.

Key words: Heterolobosea; Psalteriomonadidae; evolution; taxonomy; new species; anaerobiosis.

Introduction

The Heterolobosea is a small group of amoeboflagellates, belonging to the eukaryotic supergroup Excavata. Typical morphological features of heteroloboseans include intranuclear orthomitosis; flattened, often discoidal, mitochondrial cristae; a close association of rough endoplasmic reticulum with mitochondria; absence of stacked Golgi complexes; and movement by eruptive lobopodia in the amoeboid stage (Page and Blanton 1985). Heterolobosean flagellates have retained some distinctive excavate features (e.g. the ventral feeding groove) (Brugerolle and Simpson 2004; Park and Simpson 2011; Simpson 2003), although they

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have been reduced in some genera. In addition to morphological characteristics, all heterolobosean, except for the early-branching *Pharyngomonas kirbyi*, share a novel helix 17-1 in the secondary structure of the SSU rRNA molecule (Nikolaev et al. 2004; Park and Simpson 2011; Park et al. 2007, 2009; Wuyts et al. 2001; Yubuki and Leander 2008).

Phylogenomic analyses showed that the Heterolobosea are closely related to the Euglenozoa and the Jakobida, together forming a more inclusive clade called the Discoba or JEH (Burki et al. 2008; Hampl et al. 2009; Rodríguez-Ezpeleta et al. 2007). *Tsukubamonas globosa* was recently described as a new discobid lineage, although its precise phylogenetic position within the Discoba remains unresolved (Yabuki et al. 2011). In the last decade, the root of the eukaryotic phylogenetic tree was hypothesized to lie between the so-called "Unikonta" and "Bikonta" (Richards and Cavalier-Smith 2005; Stechmann and Cavalier-Smith 2002, 2003); however, the unikont/bikont hypothesis has fallen into disfavor (see Kim et al. 2006; Roger and Simpson 2009) and several new hypotheses regarding the position of the root of the eukaryotic phylogenetic tree were formulated. Some of them place the root specifically within the Discoba (Cavalier-Smith 2010; Gray et al. 2004; Rodríguez-Ezpeleta et al. 2007).

Despite the fact that the Heterolobosea is a relatively species-poor group (approximately 140 described species), members of the group are immensely diverse at both morphological and ecological levels. Some heteroloboseans, such as *Naegleria*, *Willaertia*, *Tetramitus, Heteramoeba*, *Euplaesiobystra*, and *Psalteriomonas*, are true amoeboflagellates that alternate between amoeboid and flagellated stages during their life cycle. The transition from the amoeba to the flagellate is incredibly fast in some lineages; for instance, this transformation in *Naegleria* takes ca. 2 hours and involves de novo formation of basal bodies with the accompanying cytoskeleton (Fulton 1993; Lee 2010). On the other hand, some members of the Heterolobosea are known to produce only flagellates or amoebae. *Acrasis* and a few of its putative relatives are sorocarpic amoebae that produce multicellular fruiting bodies (see Brown et al. 2010; Olive et al. 1983). *Naegleria fowleri* and, possibly, *Paravahlkampfia francinae* are deadly facultative parasites of humans (Visvesvara et al. 2007, 2009). *Stephanopogon* is a peculiar organism reminiscent of ciliates whose affiliation to the Heterolobosea was revealed only by detailed ultrastructure and molecular phylogenetic analyses (Cavalier-Smith and Nikolaev 2008; Lipscomb and Corliss 1982; Parfrey et al. 2010; Patterson and Brugerolle 1988; Yubuki and Leander 2008). Although most heteroloboseans live in sediments and feed on bacteria, the ecological diversity of the group is vast as well. In comparison with the other eukaryotic groups, many members of the Heterolobosea flourish in extreme environments such as acidic, hot, and hypersaline conditions (Amaral Zettler et al. 2002; Baumgartner et al. 2009; De Jonckheere et al. 2009; Guzmán-Fierros et al. 2008; Park et al. 2007, 2009; Park and Simpson 2011; Sheehan et al. 2003).

Anaerobic/microaerophilic heteroloboseans constitute another such ecological group living under harsh conditions. At least two independent lineages have been discovered. The first one is represented by the extreme halophile *Pleurostomum flabellatum* which grows at low oxygen concentrations and appears to lack mitochondrial cristae (Park et al. 2007). The second lineage is more diverse and includes *Monopylocystis visvesvarai*, *Psalteriomonas lanterna*, and *Sawyeria marylandensis* (Broers et al. 1990; Nikolaev et al. 2004; O'Kelly et al. 2003). Mitochondria of *Sawyeria* and *Psalteriomonas* have been reduced to hydrogenosomes (Barberà et al. 2010; de Graaf et al. 2009). In addition, *Vahlkampfia anaerobica*, *Lyromonas vulgaris*, and *Percolomonas descissus* have been described from habitats with low oxygen concentrations (Bernard et al. 2000; Broers et al. 1993; Brugerolle and Simpson 2004; Smirnov and Fenchel 1996). However, their phylogenetic position is unclear due to the lack of DNA sequence data. The general metabolic potential of heteroloboseans has become complicated by the recently published genome project of *Naegleria gruberi* (Fritz-Laylin et al. 2010). It appears that this presumably bona fide aerobe with typical mitochondrial morphology may be capable switching to anaerobic metabolism under hypoxia. Therefore, anaerobic life styles (or facultatively anaerobic life styles) may be more common in heteroloboseans than expected.

The systematics of heteroloboseans is chaotic due to (1) absence of reliable morphological features, (2) unclear internal phylogeny, and (3) non-unified concepts for the main heterolobosean subgroups. Even the definition of the Heterolobosea itself is confusing and two different taxonomic concepts exist. The first concept, emphasized by Park and Simpson (2011), considers the Heterolobosea as a monophyletic taxon containing organisms with trophic life stages as amoebae-only, amoeboflagellates, and flagellates-only and includes diverse taxa such as *Acrasis*, *Naegleria*, *Neovahlkampfia*, *Tetramitus*,

Stephanopogon, *Pharyngomonas*, and *Psalteriomonas*. In contrast, Cavalier-Smith (1993b) and Cavalier-Smith and Nikolaev (2008) refer to this inclusive group as the "Percolozoa" and consider the 'Heterolobosea' as a paraphyletic subgroup of taxa that were historically classified as amoebae. We concur with Nikolaev et al. (2004) who suggested that "the term Percolozoa should probably become the junior synonym of the term Heterolobosea" and recognize the broader and monophyletic concept of the Heterolobosea in this paper (Park and Simpson 2011).

Heterolobosean genera have been classified into 8 families: Acrasidae Van Tieghem, 1880, Vahlkampfiidae Jollos, 1917, Stephanopogonidae Corliss, 1961, Gruberellidae Page and Blanton, 1985, Psalteriomonadidae Cavalier-Smith, 1993, Lyromonadidae Cavalier-Smith, 1993, Percolomonadidae Cavalier-Smith and Nikolaev, 2008, and Pharyngomonadidae Cavalier-Smith and Nikolaev, 2008. The largest family Vahlkampfiidae Jollos, 1917 is paraphyletic and the other families, except for the Pharyngomonadidae, branch from within it. Although reclassification of the Vahlkampfiidae by splitting it into more monophyletic groups is necessary, the data for doing so are currently equivocal.

Although heteroloboseans are highly important from evolutionary and ecological points of view, their diversity has been considerably understudied. The aim of the present study is to thoroughly examine the diversity of anaerobic heteroloboseans. We have isolated 31 heterolobosean strains from freshwater, brackish-water and marine anoxic/microoxic habitats, and studied their light-microscopic morphology and phylogenetic position as inferred from the analysis of SSU rDNA sequences. We also examined the cell ultrastructure of two isolates. We recognized each species based on either a distinct morphology, placement in the SSU rDNA tree or a combination of both criteria. We showed that the diversity of anaerobic heteroloboseans is richer than previously expected and that non-halophilic anaerobic heteroloboseans, such as *Percolomonas descissus* and *Vahlkampfia anaerobica*, constitute a single clade, the Psalteriomonadidae. In this paper, we described three new species and two new genera, and revised the taxonomy of the Psalteriomonadidae.

Results

General Observations

Information on the origin of strains included in the present study is summarized in Table 1. Cells of all strains were found at the bottom of the culture tubes in the presence of bacteria and slowly died when exposed to oxygen near the upper part of the tubes, suggesting that the cells were anaerobic or microaerophilic. Only amoebae (*Psalteriomonas* spp., *Sawyeria marylandensis*) or flagellates (*Monopylocystis visvesvarai*, *Harpagon* spp., *Pseudoharpagon pertyi*) were present in the cultures; no sign of transformation of flagellates into amoebae or vice versa, and no cysts were observed during ca. 200 passages of the cultures. The morphology of amoebae was characteristic for the Heterolobosea, i.e. they were of the "limax" type, with an anterior, eruptive hyaline front, and locomotive forms were monopodial. No floating forms were observed. All amoebae were isolated from freshwater sediments. The amoebae of each species differed in size and in the presence/absence of hydrogenosomal aggregates. Flagellates were isolated from freshwater, brackish and marine sediments. They were always quadriflagellated with acronematic flagella and possessed a conspicuous ventral feeding groove. The flagella inserted subapically at the top of the groove, parallel to each other, and were directed ventroposteriorly. They beat together, close to the groove. Cells rotated when swimming. The nucleus was subapical. The flagellates of the particular species differed in the size and shape of the ventral groove, relative lengths of flagella, and degree of pleomorphism.

Light-microscopic Species Observations

Psalteriomonas lanterna

The amoebal stage of freshwater strains CIZOV2, VIT3, VIT5, VIT9, and VT2 corresponded to the original description by Broers et al. (1990), except for the structure of nucleus (Fig. 1). Unlike the type strain of *P. lanterna* whose amoebae were reported to possess a single central nucleolus, the cells of our strains possessed one or two parietal nucleoli located opposite each other in the nucleus. A hydrogenosomal aggregate was present close to the nucleus (Figs 1, 4E, F). The uroid was smooth, without filaments. Cells of the strain VIT3 were 47.5 ± 7.1 (32.1 – 63.7) μm long and 12.2 ± 2.2 (7.2 – 18.0) μm wide, with the mean length/width ratio 4.1. Cells of the strain VIT5 were 44.3 ± 6.7 (31.4 – 60.2) μm long and 10.1 ± 1.9 (6.8 – 14.1) μm wide, with the mean length/width ratio 4.5. Cells of the strain VT2 were 47.5 ± 8.1 (28.9 – 63.1) μm long and 10.8 ± 2.0 (7.1 – 15.1) μm wide, with the mean length/width ratio 4.5. The mean cell dimensions of the three strains of *P. lanterna* were 46.4 ± 7.4

Table 1. List of strains included in the study. [1]same isolate as in Kolisko et al. (2008) and Cepicka et al. (2010).

Species	Isolate	Locality	Coordinates	Habitat	GenBank acc. no.
Harpagon descissus	AMT	Tambopata NNR, Peru	12°49′S, 69°16′W	Fresh-water sediment	JN606327
	BAUM	Baum pond, Fayetteville, AR, USA	36°05′N, 94°18′W	Fresh-water sediment	JN606328
	IND2	Bhangarh, India	27°05′N, 76°17′E	Fresh-water sediment	JN606329
	KG3N	valley close to Engilchek village, Kyrgyzstan	41°57′N, 79°18′E	Fresh-water sediment	JN606330
	KOMPKOJ[1]	Kojčice, Czech Republic	49°28′N, 15°15′E	Compost pile	JN606331
	PANT1	Pantanal NP, Brazil	16°37′S, 56°44′W	Fresh-water sediment	JN606332
	SOOS1	Soos NNR, Czech Republic	50°08′N, 12°24′E	Fresh-water sediment	JN606333
	TEXEL	Texel island, Netherlands	53°01′N, 04°44′E	Fresh-water discharge ditch	JN606334
	TOCOV	Former village Tocov, Czech Republic	50°18′N, 13°05′E	Fresh-water sediment	JN606335
	VT1	Velký Tisý pond, Czech Republic	49°03′N, 14°43′E	Fresh-water sediment	JN606336
	WS	Lake Wilson stream, Fayetteville AR, USA	36°01′N, 94°14′W	Fresh-water sediment	JN606337
Harpagon schusteri	CIZOV1	Čížov, Czech Republic	48°52′N, 15°52′E	Fresh-water sediment	JN606338
	IND8	Bhangarh, India	27°05′N, 76°17′E	Fresh-water brook	JN606339
	INDSIP	Boroda dam, India	27°02′N, 76°15′E	Fresh-water sediment	JN606340
	OLB4	Olbasee lake, Germany	51°16′N, 14°35′E	Fresh-water sediment	JN606341
	TUN2	Chomutov, Czech Republic	50°27′N, 13°21′E	Fresh-water sediment	JN606342
	VITSED	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606343
Monopylocystis visvesvarai	PC4BIC	Peggy's Cove, Canada	44°29′N, 63°55′W	Marine sediment	JN606344
Psalteriomonas lanterna	CIZOV2	Čížov, Czech Republic	48°52′N, 15°52′E	Fresh-water brook	JN606345
	VIT3	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606346
	VIT5	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606347
	VIT9	Kamenice, Czech Republic	49°54′N, 14°35′E	Fresh-water sediment	JN606348
	VT2	Velký Tisý pond, Czech Republic	49°03′N, 14°43′E	Fresh-water sediment	JN606349
Psalteriomonas magna	CERETE	Cereté, Columbia	08°53′N, 75°47′W	Fresh-water sediment	JN606350
	IND7	Bhangarh, India	27°05′N, 76°17′E	Fresh-water brook	JN606351
	KIZILLAR	Kizillar, Turkey	37°31′N, 35°42′E	Fresh-water sediment	JN606352
Sawyeria marylandensis	ITZAVL	Italy	n.a.	Fresh-water irrigation canal	JN606353
	LUH2	Alluvial plain of Vltava river, Czech Republic	48°48′N, 13°57′E	Fresh-water sediment	JN606354
	LUH3[1]	Alluvial plain of Vltava river, Czech Republic	48°48′N, 13°57′E	Fresh-water sediment	JN606355
Pseudoharpagon pertyi	EVROS2	Evros delta, Greece	40°48′N, 26°01′E	Brackish sediment	JN606356
	NY0199	Bamfield, Canada	48°82′N, 125°12′W	Marine sediment	JN606357

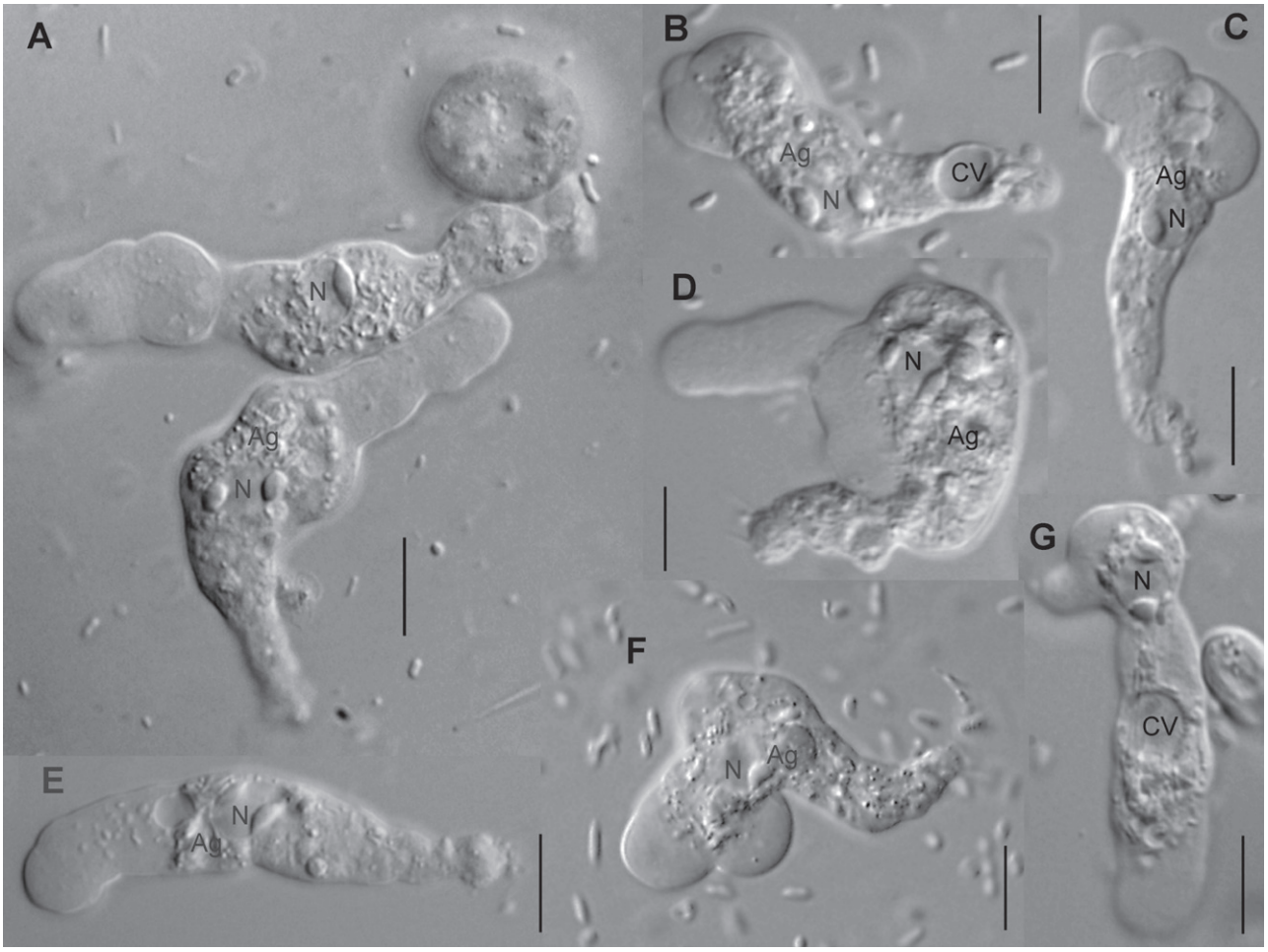

Figure 1. Living cells of *Psalteriomonas lanterna* strains VIT3 (**A, E**) and VT2 (**B – D, F – G**). Ag – aggregate of hydrogenosomes; CV – contractile vacuole; N – nucleus. Bar = 10 μm.

(28.9 – 63.7) μm × 11 ± 2.2 (6.8 – 18.0) μm, with the mean length/width ratio 4.4. Flagellate and cyst forms were not observed.

Psalteriomonas magna sp. nov.

The amoebal stage of freshwater strains KIZILLAR, CERETE, and IND7 was bigger than that of *P. lanterna* (Fig. 2). Cells of the strain KIZILLAR were 72.2 ± 11.8 (49.9 – 102.3) μm long and 16.4 ± 4.0 (8.1 – 30.0) μm wide, with the mean length/width ratio 4.6. Cells of the strain CERETE were 65 ± 9.1 (50.1– 90.8) μm long and 14.7 ± 2.3 (10.2 – 19.0) μm wide, with the mean length/width ratio 4.5. Cells of the strain IND7 were 66.2 ± 8.6 (40.1 – 82.9) μm long and 14.9 ± 2.4 (10.0 – 23.1) μm wide, with the mean length/width ratio 4.6. The mean cell dimensions of the three strains of *P. magna* were 67.8 ± 10.4 (40.1 – 102.3) × 15.3 ± 3.1 (8.1 - 30.0) μm, with the mean length/width ratio 4.5. An aggregate of hydrogenosomes was situated close to the nucleus (Figs 2, 4). The nucleus contained one or two parietal nucleoli located opposite each other in the nucleus (Fig. 2). Long uroidal filaments were observed in some cells (Fig. 2C, E, H). Flagellate and cyst forms were not observed.

Sawyeria marylandensis

The amoebal stage of freshwater strains ITZAVL, LUH2, and LUH3 corresponded to the original description of *Sawyeria marylandensis* (O'Kelly et al. 2003), (Fig. 3). Cells of the strain ITZAVL were 41.1 ± 11.9 (17.1 – 69.8) μm long and 9.5 ± 2.0 (5.9 – 14.3) μm wide, with the mean length/width ratio 4.4. Cells of the strain LUH3 were 34.0 ± 6.3 (22.0 – 51.5) μm long and 9.6 ± 2.5 (5.4 – 16.7) μm wide, with the mean length/width ratio 3.7. The mean cell dimensions of the two strains of *S. marylandensis* were 37.6 ± 10.1 (17.1 – 69.8) × 9.5 ± 2.3 (5.4 – 16.7) μm, with the mean cell length/width ratio 4.0. The nucleus of strain ITZAVL contained

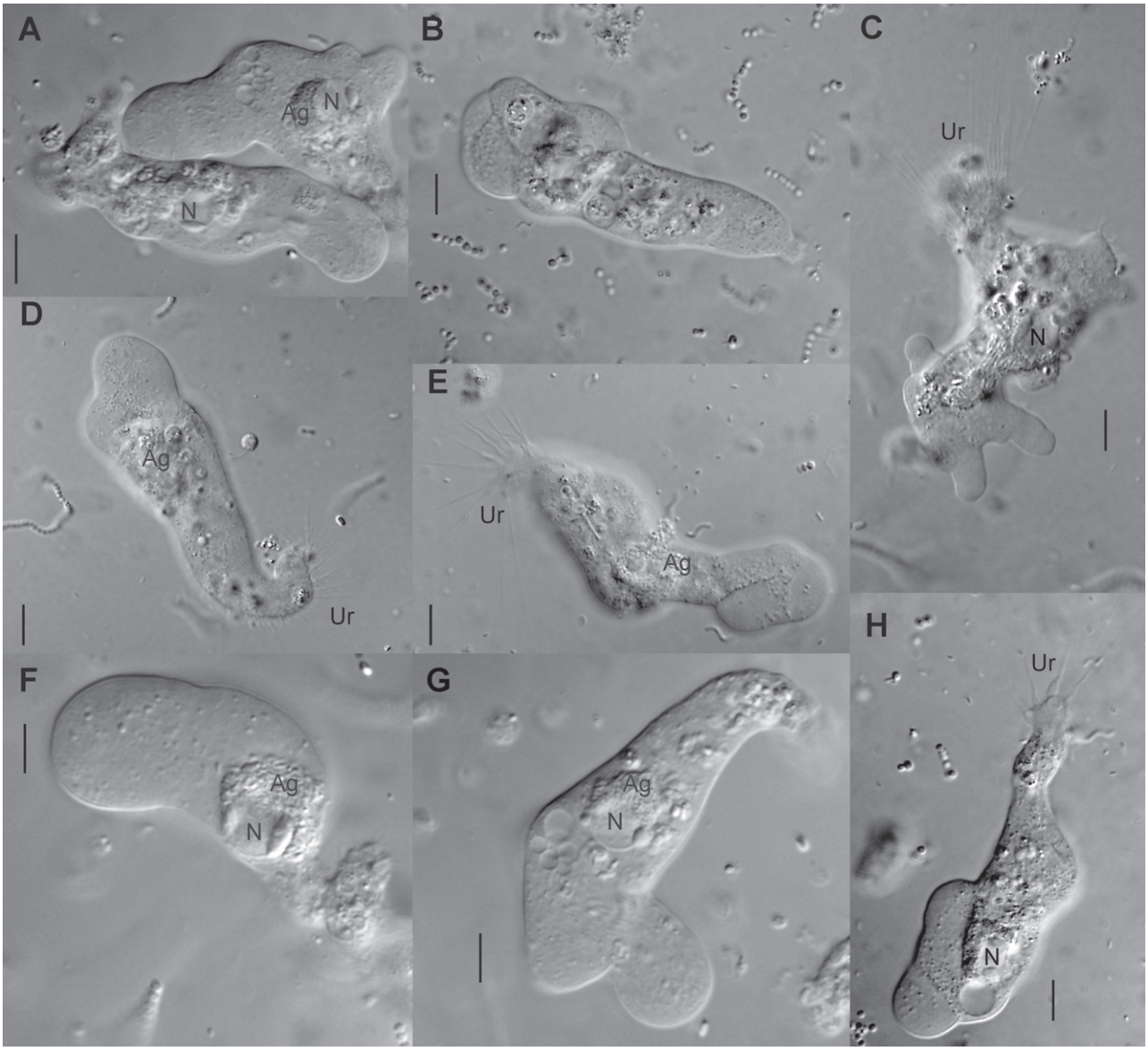

Figure 2. Living cells of *Psalteriomonas magna* strains KIZILLAR (**A – D, F, G**) and IND 7 (**E, H**). Ag – aggregate of hydrogenosomes; N – nucleus; Ur – uroidal filaments. Bar = 10 μm.

two parietal nucleoli situated opposite each other (Fig. 3B, C, D), whereas strain LUH3 possessed several parietal nucleoli (Fig. 3E – G). No aggregate of hydrogenosomes was observed (Fig. 4G, H). Flagellate and cyst forms were not observed.

The Flagellate Stage of *Monopylocystis visvesvarai*

The flagellate stage of the marine strain PC4BIC was elongated and relatively uniform in shape and size (Figs 5, 9F – J). The posterior end of the cell was either rounded or pointed. The cell length was 15.7 ± 1.7 (11.1 – 19.9) μm. A slightly spiral prominent ventral groove started anteriorly and extended almost to the posterior end of the cell (Figs 5C, D, E, G, 9H). The four flagella were unequal; the longest flagellum was long up to 1.5 times body length, two other flagella were more or less shorter than the longest flagellum and the fourth flagellum was the shortest one. Many cells adhered to the substrate by the distal ends of their four flagella. Dividing protargol-stained cell with two nuclei and two pairs of flagella was observed (Fig. 9I). Amoeboid and cyst stages were not observed.

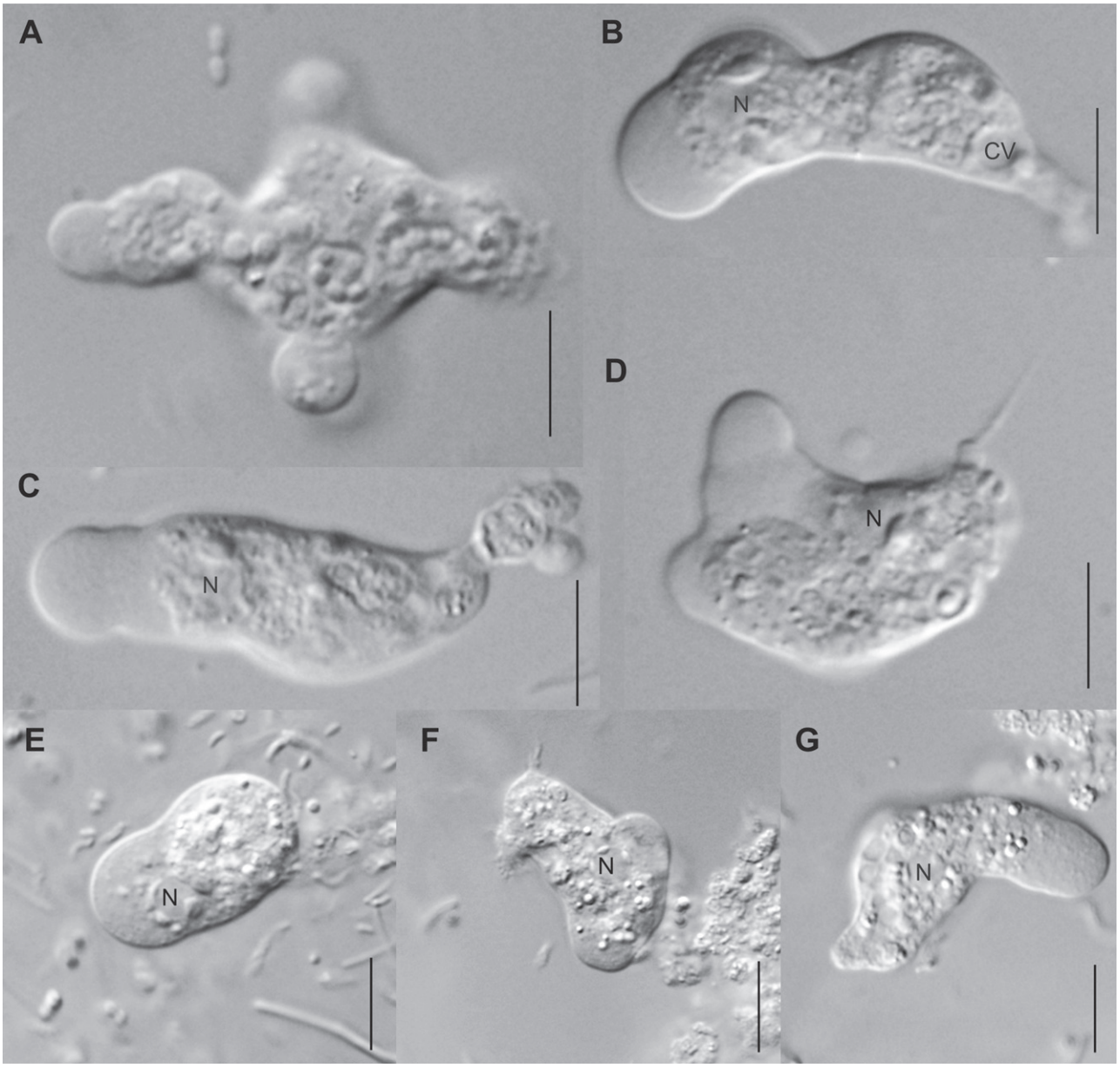

Figure 3. Living cells of *Sawyeria marylandensis* strains ITZAVL (**A – D**) and LUH3 (**E – G**). CV – contractile vacuole; N – nucleus. Bar = 10 μm.

Harpagon descissus comb. nov.

The flagellate stage of *H. descissus* freshwater strains corresponded to the previous observations of *Percolomonas descissus* (Brugerolle and Simpson 2004; Klug 1936). The cells were elongated (Figs 6, 9D, E). The cell length of four *H. descissus* strains was: PANT1S – 16.8 ± 3.3 (10.5 – 24.9) μm, SOOS1 – 17.8 ± 3.8 (10.2 – 25.0) μm, TEXEL – 18.2 ± 3.6 (12.7 – 29.7) μm, and VT1 – 18.1 ± 4.9 (12.0 – 31.4) μm. The mean cell length of the four strains of *H. descissus* was 17.0 ± 4.0 (10.2 – 31.4) μm. The cells were highly variable in shape, in part associated with the nutritional status and age of the culture, and four distinct morphotypes were determined: spindle-shaped cell with a short pointed posterior end (Fig. 6D), spindle-shaped cell with elongated pointed posterior end (Fig. 6A, I), slender elongated cell (Fig. 6H, L), and pear-shaped short cell with rounded posterior end (Fig. 6B, C, E, F, K). Cells which were morphologically intermediate between particular morphotypes were also observed. Relative abundance of the

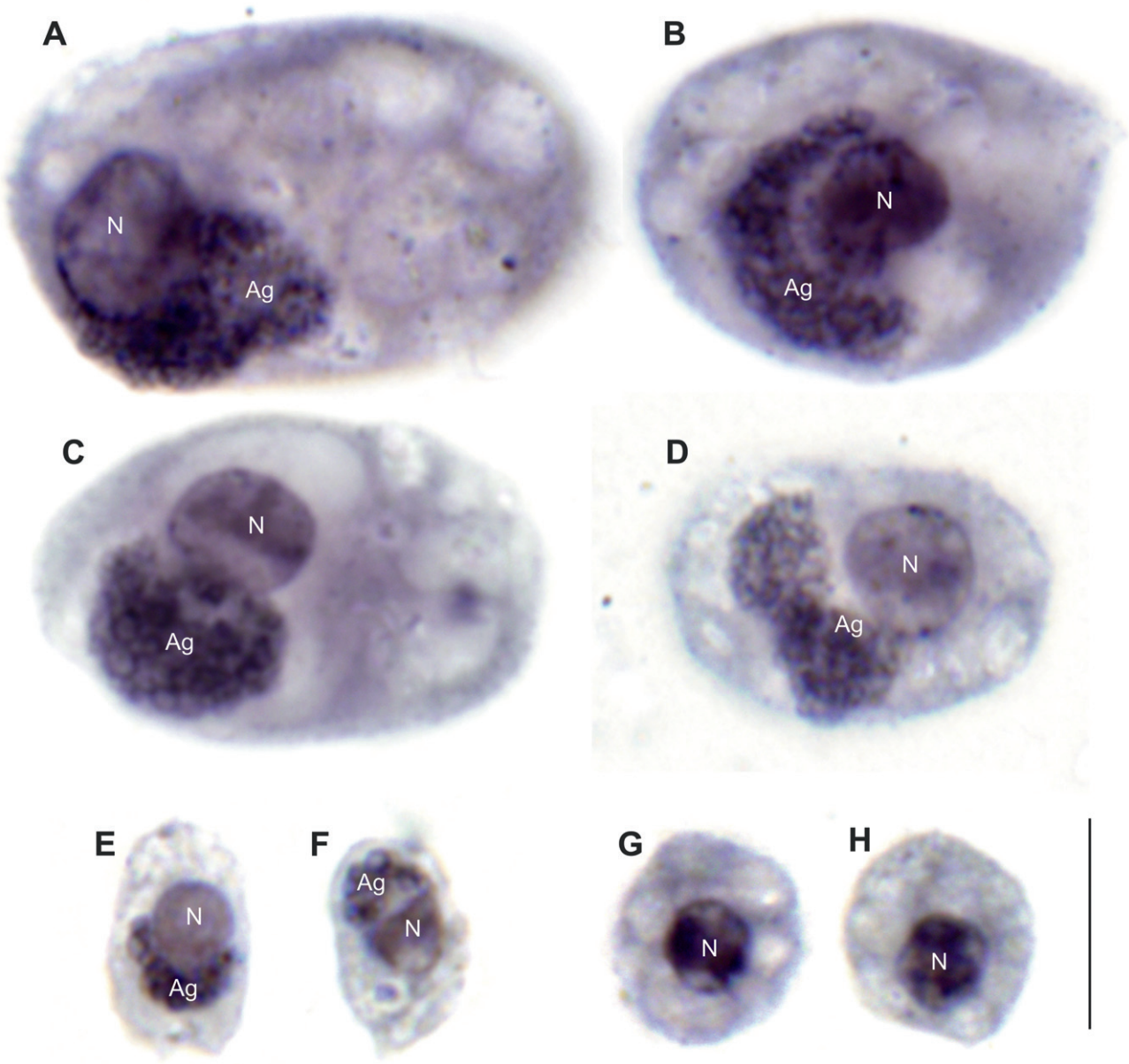

Figure 4. Protargol-stained specimens of *Psalteriomonas magna* sp. nov. strain KIZILLAR (**A – D**), *P. lanterna* strain VIT5 (**E, F**), and *Sawyeria marylandensis* strain ITZAVL (**G, H**). Ag – aggregate of hydrogenosomes; N – nucleus. Bar = 10 μm.

morphotypes varied within strains and observations, but all four morphotypes were observed in all examined strains. The ventral groove was less conspicuous than in *Monopylocystis visvesvarai* and extended to ½ of the length of the cell body in the spindle-shaped morphotype. The four flagella were unequal (Fig. 9D, E). The longest flagellum was long, at 1 – 2 times body length. The second flagellum was shorter, about 3/5 length of the longest flagellum. The remaining two flagella were equal in length and slightly shorter than the second flagellum. Some cells of *H. descissus* sp. nov. adhered to the substrate by the posterior end of the cell. A dividing cell with six flagella was observed (Fig. 6I, J). Amoebae and cysts were not observed.

Harpagon schusteri sp. nov.

The flagellate stage of *H. schusteri* sp. nov. freshwater strains were in most essential details, such as cell and flagella lengths, identical to those of

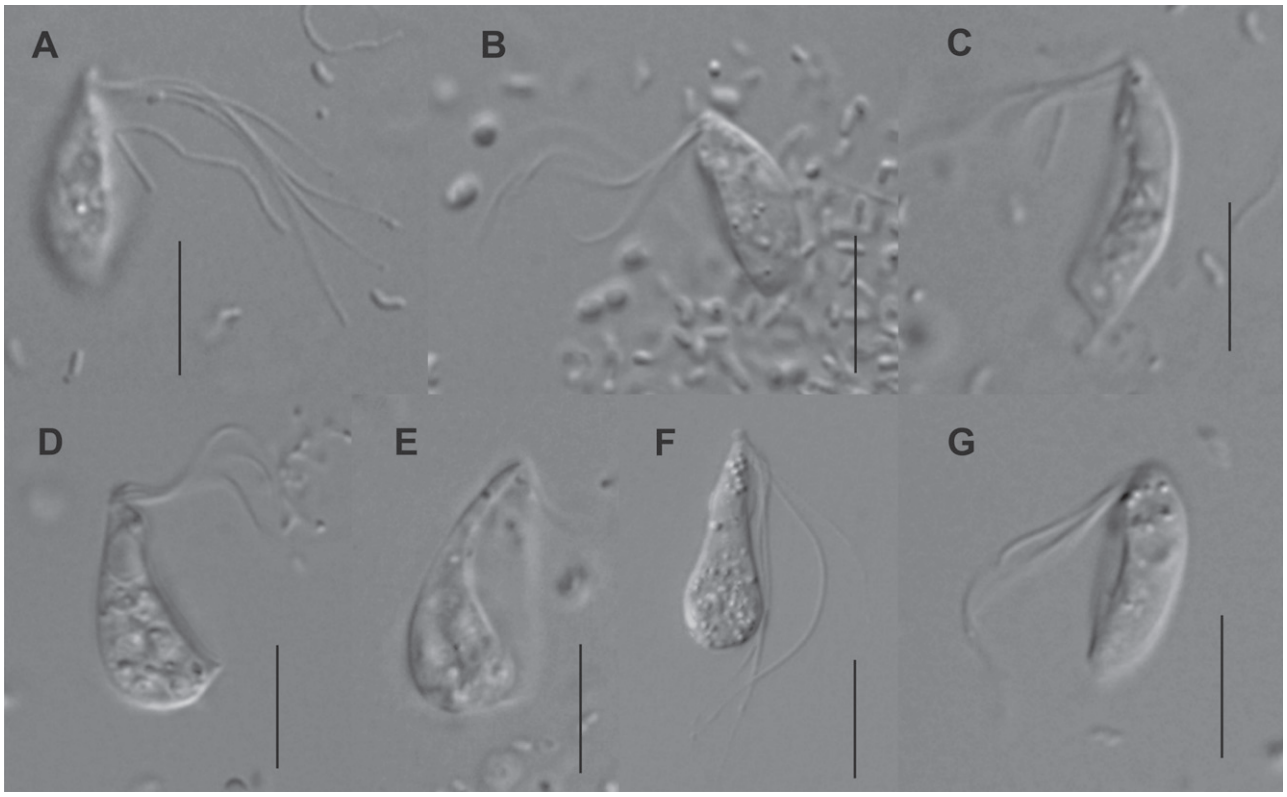

Figure 5. Living cells of *Monopylocystis visvesvarai* strain PC4BIC. Bar = 10 μm.

H. descissus (Figs 7, 9A – C). The cell length of two strains was: IND8 – 18.0 ± 3.6 (13.0 – 34.2) μm, INDSIP – 16.3 ± 3.2 (10.8 – 25.2) μm. The mean cell length of the two strains of *H. schusteri* was 17.1 ± 3.5 (10.8 – 34.2) μm. Three distinct morphotypes, the same as in *H. descissus* comb. nov., could be identified in the culture: spindle-shaped cell with a short pointed posterior end (Fig. 7G, H), slender elongated cell (Fig. 7B), and pear-shaped short cell with rounded posterior end (Fig. 7C, D, E, F). Cells with an elongated pointed posterior end were not observed in any strain. Amoebae and cysts were not observed.

Pseudoharpagon pertyi sp. nov.

The morphology of the flagellate stage from brackish and marine strains EVROS2 and NY0199 was similar to *Harpagon* spp., though the cells were slightly smaller. They were elongated and relatively uniform in shape and size (Figs 8, 9K – O). The posterior end was either rounded or pointed. The cell length of the two strains was: EVROS2 – 13.7 ± 3.5 (8.5 – 21.3) μm, NY0199 – 14.0 ± 2.0 (11.5 – 19.7) μm. The prominent ventral groove extends to ½ - 2/3 of body length. The four flagella were unequal in length. The longest flagellum was up to 1.5 times body length. The remaining three flagella were approximately equal and cell-body in length. Some cells of *P. pertyi* sp. nov. adhered to the substrate by a cytoplasmic bridge formed from the posterior end of the cell (Fig. 8B, F). A dividing protargol-stained cell with two nuclei and two pairs of flagella was observed (Fig. 9M). Amoebae and cysts were not observed. The nucleolar morphology could not be determined from our light-microscopic pictures.

Ultrastructure Observations

Flagellate Stage of *Monopylocystis visvesvarai*

The structure of the flagellar apparatus of *Monopylocystis visvesvarai* was very similar to that of *Harpagon descissus* comb. nov., *Psalteriomonas lanterna*, and *P. vulgaris* (Fig. 10A, see Broers et al. 1990, 1993; Brugerolle and Simpson 2004). All four basal bodies possessed flagella and were arranged in two pairs, #1 and #4 forming the posterior pair, and #2 and #3 forming the anterior one. There were two straight rhizoplasts (Rh_1 and Rh_2) that originated anteriorly and were closely associated. The periodicity of cross striation of the rhizoplasts was about 38 nm. The rhizoplasts connected to basal

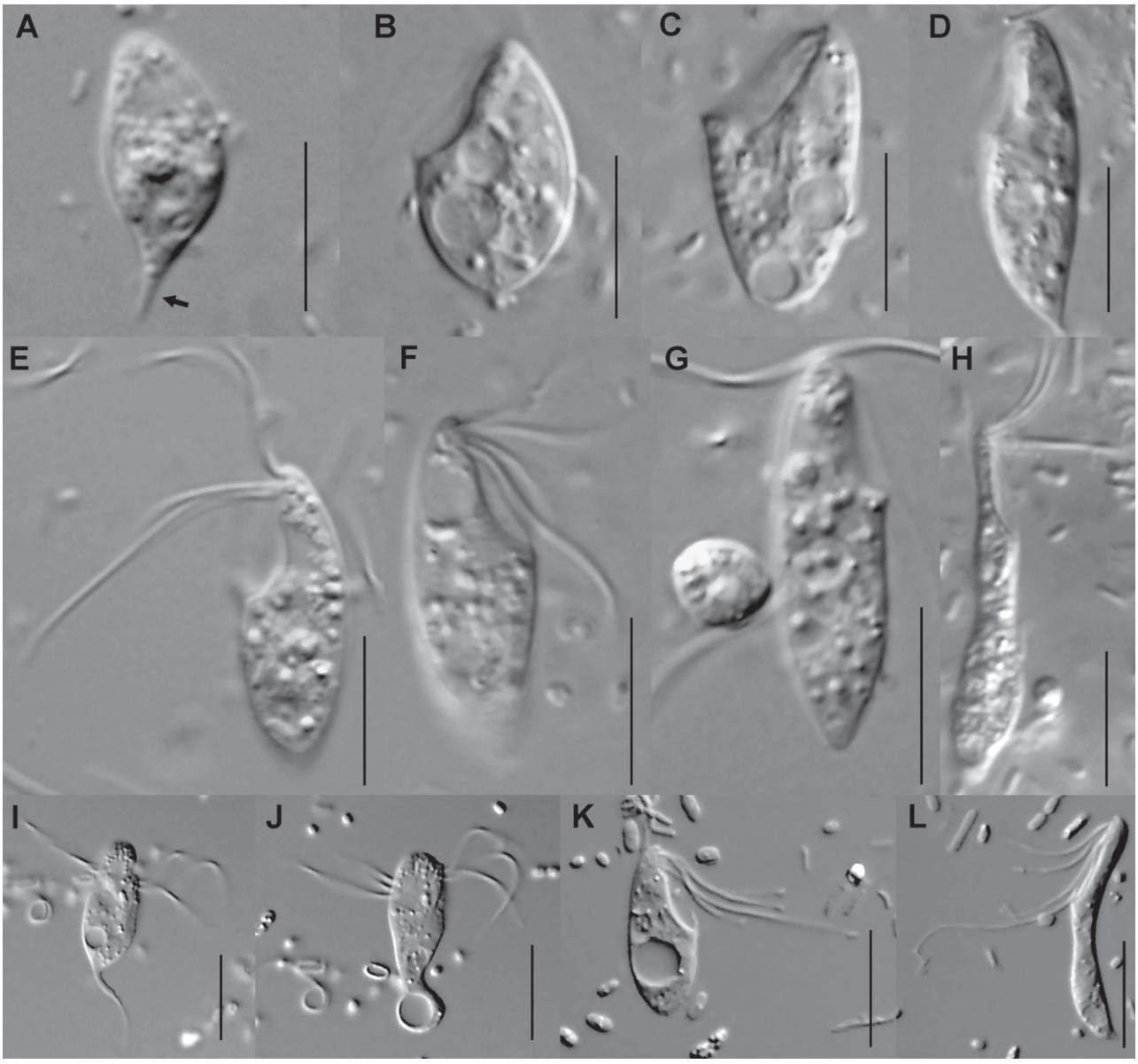

Figure 6. Living cells of *Harpagon descissus* comb. nov. strains TOCOV (**A**), TEXEL (**B – G**), VT1 (**H**), and PANT1 (**I – L**). Arrow – long spike at the posterior end. Bar = 10 μm.

bodies #2 (Rh_1) and #1 (Rh_2), respectively. Both the rhizoplasts continued posteriorly and connected to microtubular ribbon R_1. The ribbon R_1 was a prominent structure and consisted of a curved row of thick microtubules originating near basal body #1. The basal bodies were interconnected by fibres (Fi). A bundle of microfilaments (MB) connected the posterior pair of basal bodies with the concave side of R_1. MB with R_1 constituted characteristic harp-like structure.

The nucleus (N) was surrounded by rough endoplasmic reticulum (RER) and nucleolar material (Nu) formed a thin ring close to the nuclear membrane (Fig. 10B). Certain parts of the ring were thickened. No stacked Golgi apparatus was observed. Mitochondrion-related organelles (MRO) were acristate, rounded, with diameter approximately 700 – 800 nm (Fig. 10C). They were not surrounded by rough endoplasmic reticulum, but were sometimes situated close to it.

Psalteriomonas magna sp. nov.

Preliminary transmission electron microscopy of *P. magna* is presented in Figure 11. There were no traces of a flagellar apparatus in the amoeba of

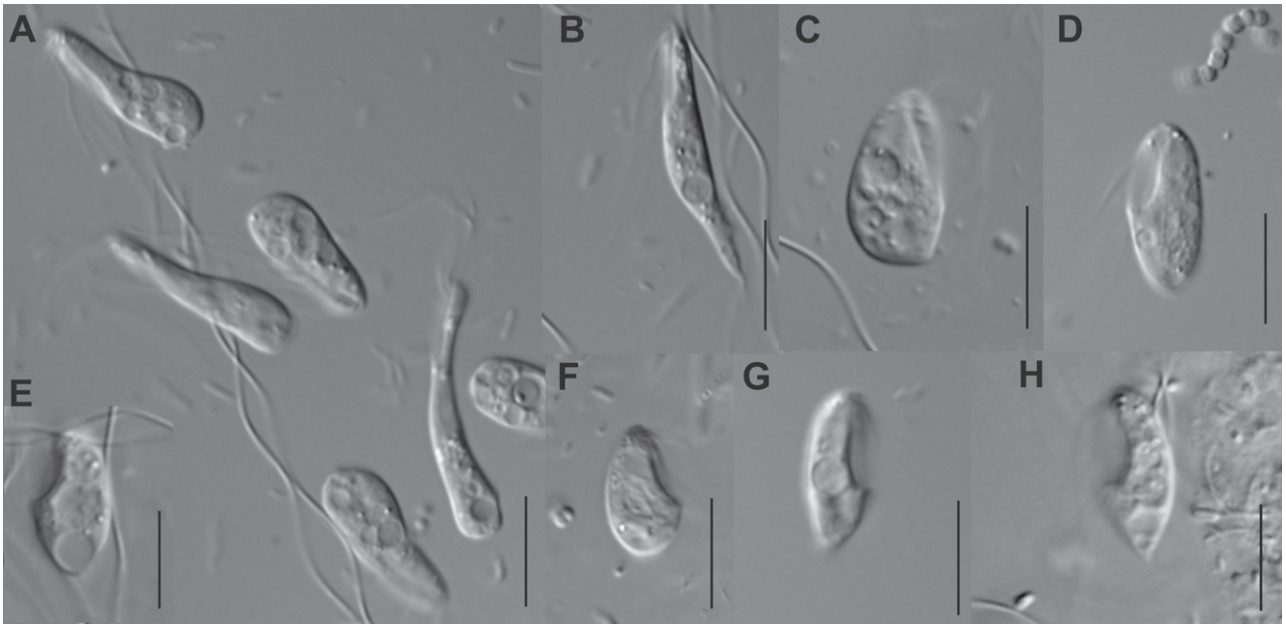

Figure 7. Living cells of *Harpagon schusteri* sp. nov. strain INDSIP. Bar = 10 μm.

the strain KIZILLAR. The nucleus (N) contained one or two parietal nucleoli (Nu) situated in nuclear lobes (Fig. 11A, C, D). No stacked Golgi apparatus was observed. Mitochondrion-related organelles (hydrogenosomes, H) were irregular in shape with a maximal diameter about 2000 nm (Fig. 11B). They were aggregated to form a cup-shaped complex that was interlaced with bacilliform bacteria (Ba; Fig. 11B, C). The prominent hydrogenosomal aggregate was situated close to the nucleus and was not surrounded by rough endoplasmic reticulum (RER). In addition to the aggregate, individual hydrogenosomes were observed in the cytoplasm of *P. magna* (IH; Fig. 11C). Although the individual hydrogenosomes seemed not to be surrounded by endoplasmic reticulum, we cannot rule out the possibility that fixation artifacts occurred.

Molecular Phylogenetic Analyses

The phylogenetic tree of the Heterolobosea as inferred from SSU rDNA is shown in Figure 12. A monophyletic group of heteroloboseans was recovered by both maximum likelihood and Bayesian methods with strong statistical support. The earliest diverging lineage of the Heterolobosea was formed by *Pharyngomonas kirbyi*. The rest of the Heterolobosea (Tetramitia sensu Cavalier-Smith and Nikolaev 2008) was split into six robust lineages whose interrelationships were unresolved: I. Genus *Neovahlkampfia* and related undetermined heteroloboseans LC103 and AND9. II. Genera *Acrasis*, *Solumitrus* and *Allovahlkampfia*, and undetermined heteroloboseans BA, OSA and AND12. Since the genetic distance between *Solumitrus* and *Allovahlkampfia* was relatively low and the observed morphological differences were minute (see Anderson et al. 2011), the genus *Solumitrus* should be synonymized with *Allovahlkampfia* in the future. III. Genera *Naegleria*, *Pleurostomum*, *Tulamoeba*, *Willaertia*, and *Marinamoeba*. IV. Genera *Percolomonas* and *Stephanopogon*. V. Genera *Vahlkampfia* and *Tetramitus*. VI. Genera *Paravahlkampfia*, *Heteramoeba*, *Euplaesiobystra*, *Vrihiamoeba*, *Oramoeba*, *Stachymoeba*, undetermined heteroloboseans RT5in38 and WIM43, and the Psalteriomonadidae. A clade formed by *Vrihiamoeba*, *Oramoeba*, *Stachyamoeba*, and the environmental sequence WIM43 was sister to Psalteriomonadidae, though without statistical support. The Psalteriomonadidae was monophyletic with high support and split into three robust subclades that were largely unresolved with respect to each other. The first subclade was formed by the genus *Pseudoharpagon* gen. nov. Genetic distance (uncorrected p-distance) between strains EVROS2 and NY0199 of *P. pertyi* sp. nov. was 0.189. The second subclade was formed by genera *Sawyeria* and *Psalteriomonas*, and two environmental sequences FN865111 and FN865530. The relationships within the second subclade were well resolved. The mean genetic distance between the two *Psalteriomonas* species was 0.049. The intraspecific genetic distance within

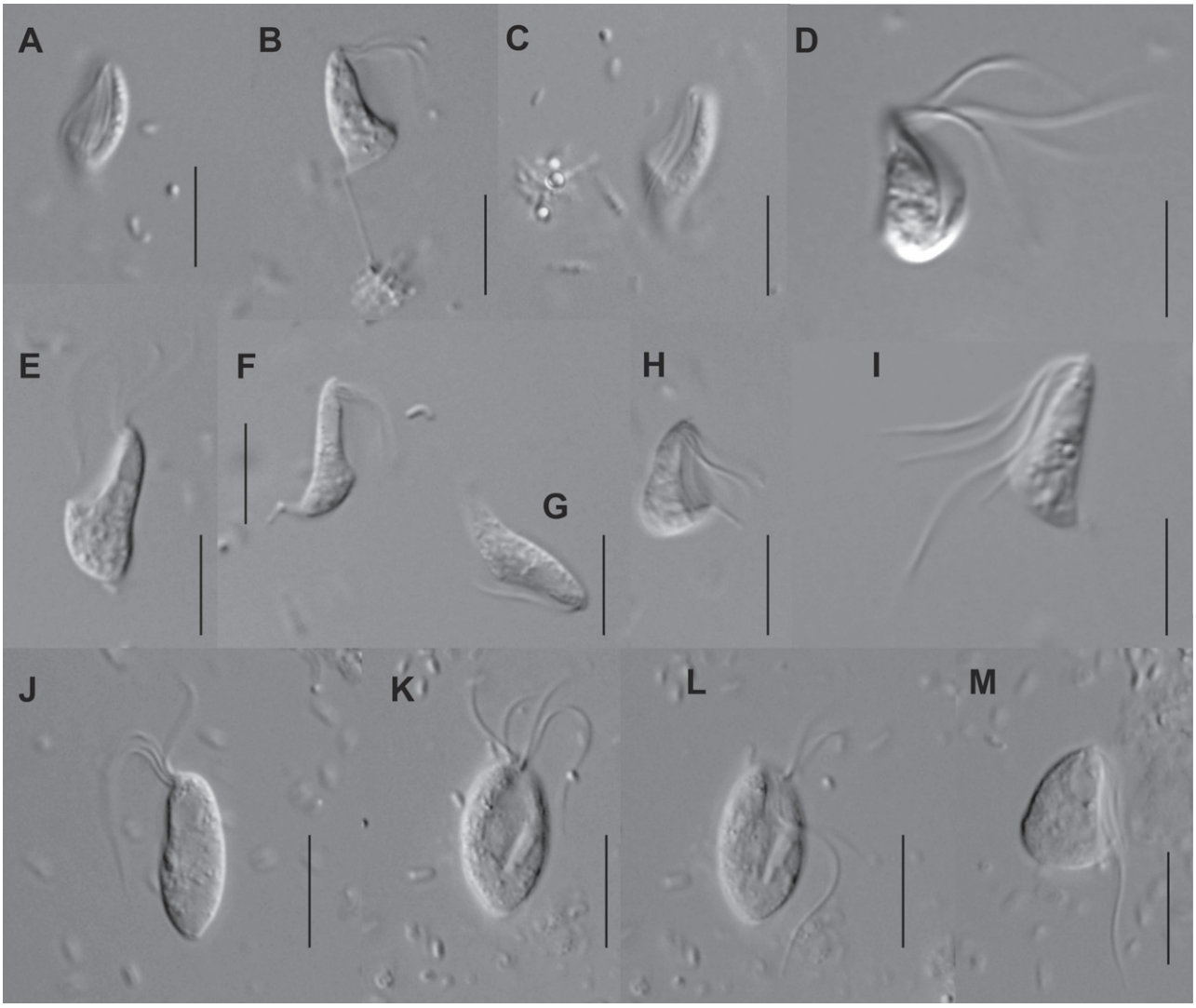

Figure 8. Living cells of *Pseudoharpagon pertyi* sp. nov. strain EVROS2 (**A – I**) and NY0199 (**J – M**). Bar = 10 μm.

Psalteriomonas lanterna reached 0.015. The intraspecific distances within *P. magna* sp. nov. and within *Sawyeria marylandensis* were negligible reaching less than 0.001. The third subclade of Psalteriomonadidae was formed by genera *Monopylocystis* and *Harpagon* gen. nov., and by the environmental sequence AF011462. The mean genetic distance between *Harpagon descissus* comb. nov. and *H. schusteri* sp. nov. was 0.127 (235 bp). Intraspecific genetic distances within *Harpagon descissus* comb. nov. and *H. schusteri* sp. nov. reached 0.036 (67 bp) and 0.024 (45 bp), respectively. The genetic distance between the type strain of *Monopylocystis visvesvarai* and strain PC4BIC was 0.004.

Discussion

Species Identity of the Isolates

The Heterolobosea is a very diverse group of protists, and different stages in the life cycle do not have the same significance in species determination. Diversity in cyst morphology, when known, may be most important in the discrimination of morphospecies. By contrast, the amoebae of many heteroloboseans share the same gross morphology and are of limited value in species identification. Due to the lack of morphological variability in heterolobosean amoebae, unrelated genera with the same cyst morphology

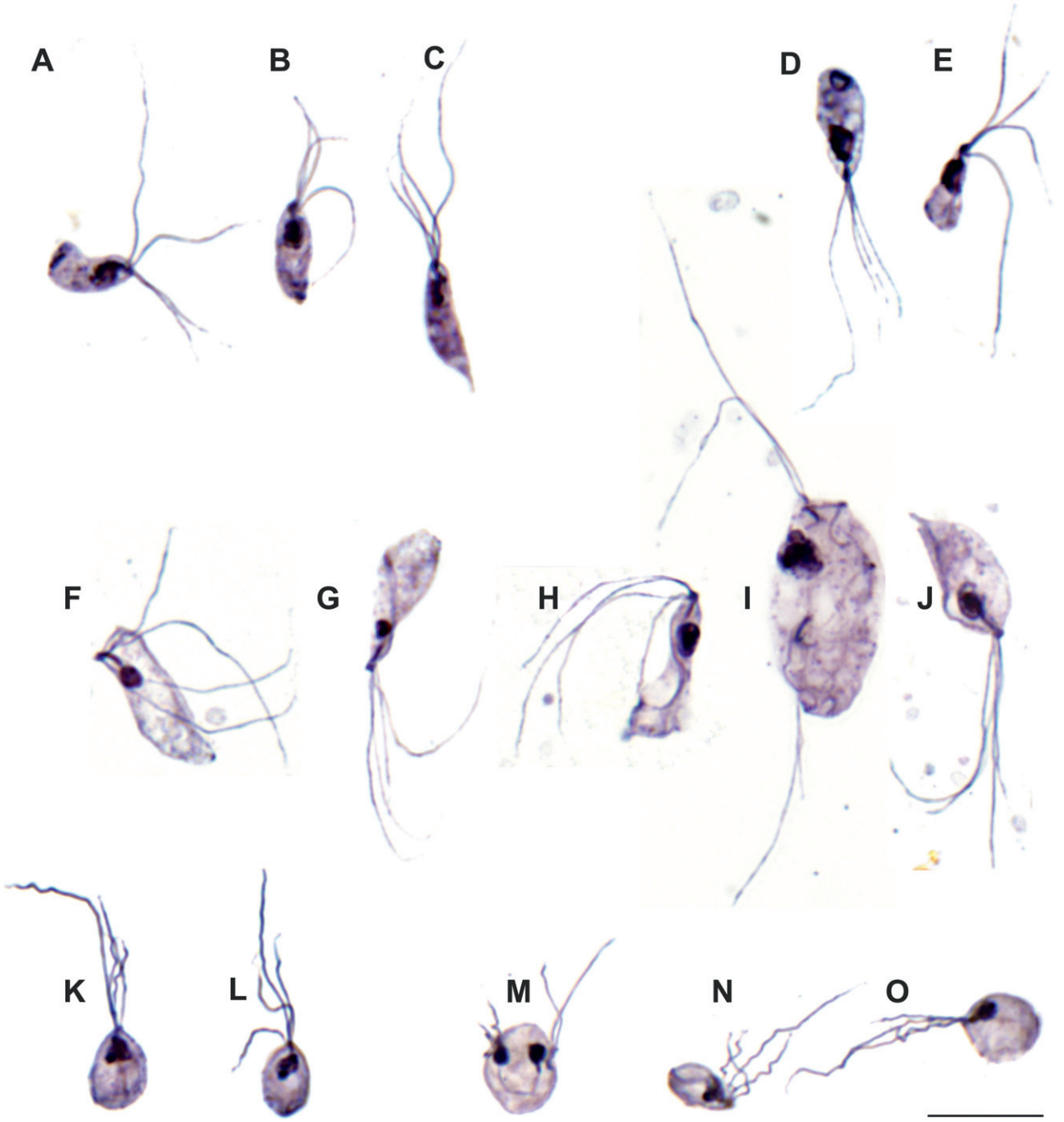

Figure 9. Protargol-stained specimens of *Harpagon schusteri* sp. nov. strain INDSIP (**A – C**), *H. descissus* comb.nov. strain TEXEL (**D, E**), *Monopylocystis visvesvarai* strain PC4BIC (**F – J**; dividing cell in **I**), and *Pseudoharpagon pertyi* sp. nov. strain EVROS2 (**K – O**; dividing cell in **M**). Bar = 10 μm.

(e.g. *Vahlkampfia*, *Neovahlkampfia*, *Allovahlkampfia*, and *Paravahlkampfia*) must be distinguished solely on the basis of their molecular-phylogenetic distances. Heterolobosean flagellates are more diverse than the amoebae. Although ultrastructural studies have shown that the mastigont of flagellates bear many morphological features important for taxonomy, most heterolobosean flagellates are considerably understudied and their ultrastructure is unknown. The number of flagella is variable at both the species and genus levels (Darbyshire et al. 1976), but most often the cells are

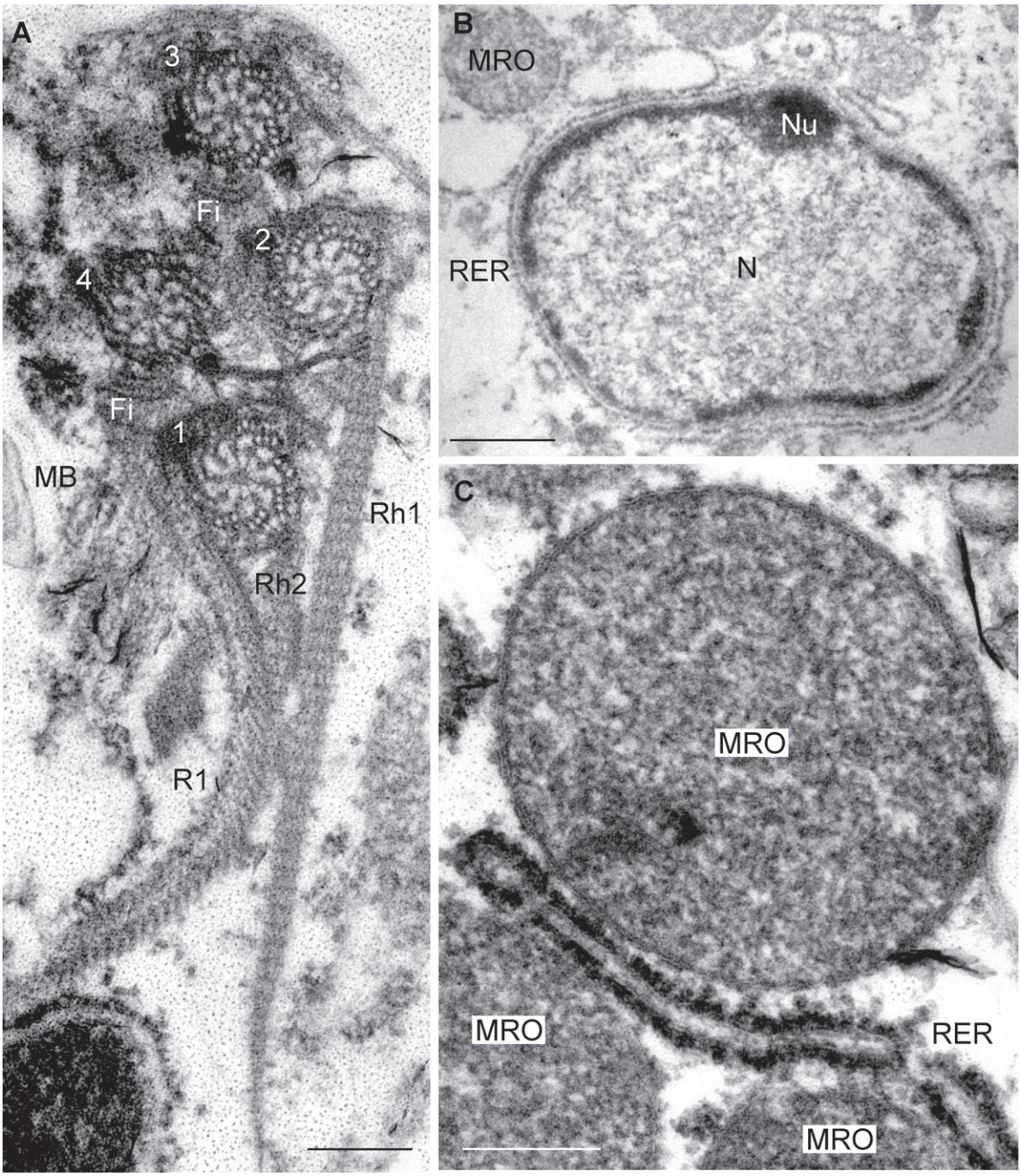

Figure 10. Ultrastructure of the flagellate of *Monopylocystis visvesvarai* strain PC4BIC. **A**, mastigont; **B**, nucleus surrounded by rough endoplasmic reticulum; **C**, mitochondrion-related organelle. 1, 2, 3, 4 – basal bodies; Fi – Fibres interconnecting the basal bodies; MB – bundle of microfilaments; MRO – mitochondrion-related organelle; N – nucleus; Nu – nucleolus; R1 – microtubular ribbon R_1; RER – rough endoplasmic reticulum; Rh1, Rh2 – rhizoplasts. Bar = 200 nm for A, C, 500 nm for B.

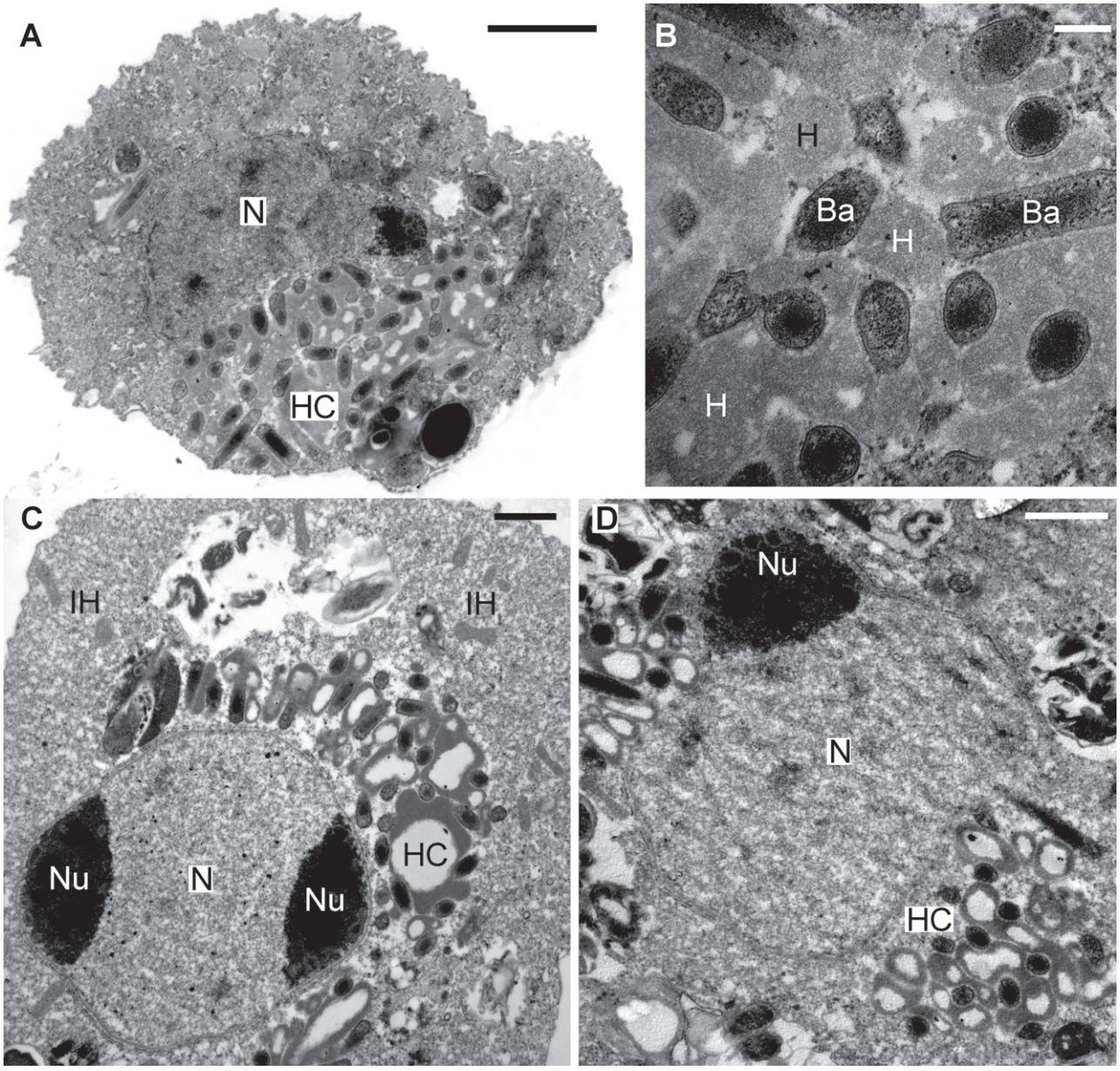

Figure 11. Ultrastructure of *Psalteriomonas magna* sp. nov. strain KIZILLAR. **A**, whole cell; **B**, detail of hydrogenosomal complex with associated bacteria; **C**, detail of the nucleus with two parietal nucleoli, hydrogenosomal complex and individual hydrogenosomes in the cytoplasm; **D** – detail of the nucleus surrounded by the hydrogenosomal complex. Ba – endosymbiotic bacterium; H – hydrogenosomes in the hydrogenosomal complex; HC – hydrogenosomal complex; IH – individual hydrogenosomes; N – nucleus; Nu – nucleolus. Bar = 2 μm for A, 250 nm for B, 1 μm for C and D.

biflagellate or quadriflagellate. The morphology of the cytostome is also variable. *Tetramitus rostratus* and *Pleurostomum flabellatum* flagellates possess a conspicuous rostrum and a tubular cytostome (Park et al. 2007; Patterson et al. 2000) while flagellates of some other species have a broad longitudinal feeding groove and lack a rostrum. In addition, the cytostome is reduced in some genera, e.g. in *Naegleria* and *Willaertia* (Page 1988; Robinson et al. 1989). Because the life cycle of many species has not been thoroughly examined, it is theoretically possible to inadvertently describe two life stages of one organism as different species.

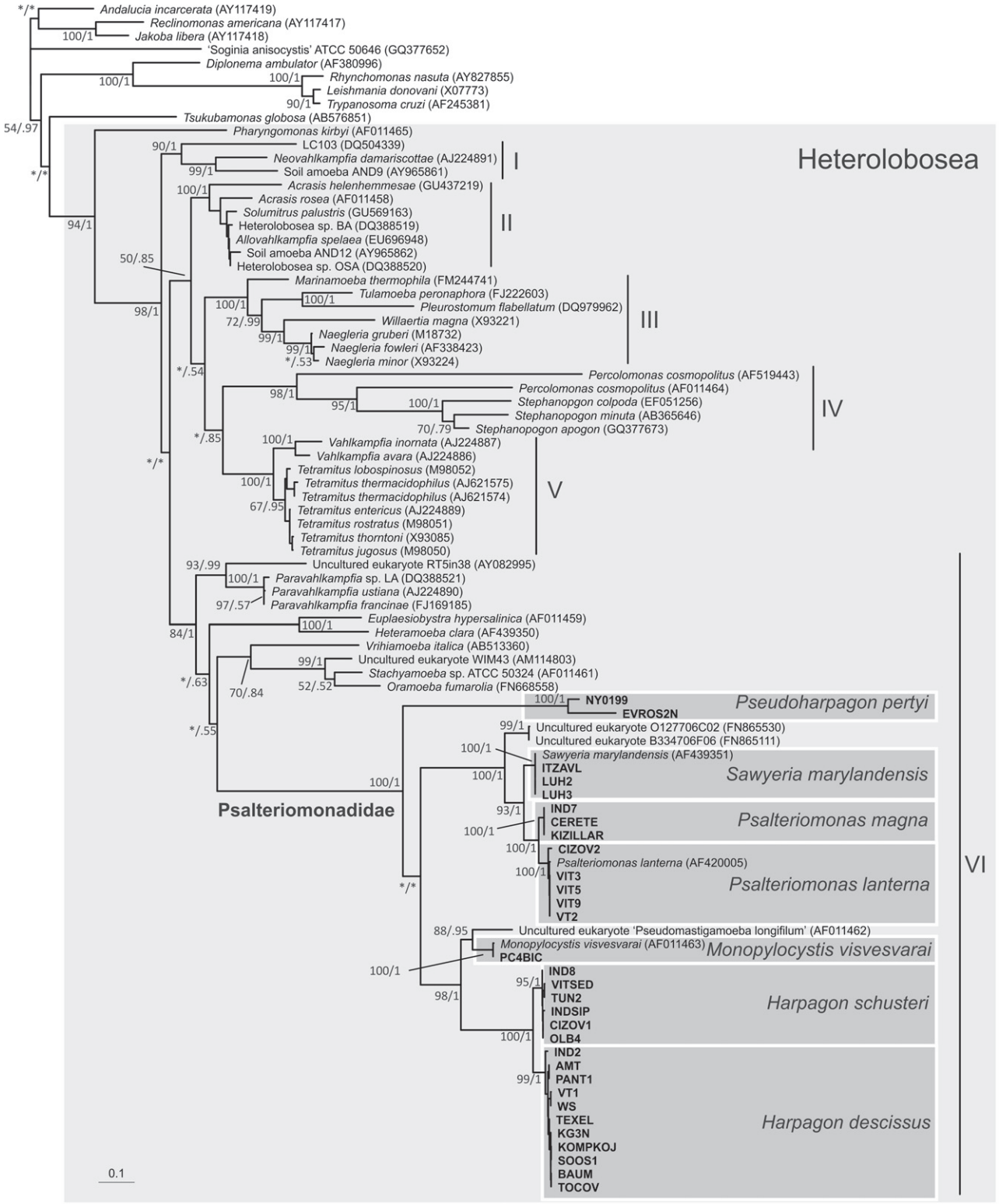

Figure 12. Phylogenetic tree of Heterolobosea based on SSU rDNA. The tree was constructed by the maximum likelihood method in RAxML (GTRGAMMAI model) and was rooted with representatives of other Discoba lineages. The values at the nodes represent statistical support in bootstrap values (RAxML)/posterior probabilities (MrBayes). Support values below 50%/.50 are represented by an asterisk (*). Six main clades of Tetramitia are labeled.

A molecular species concept based on the ITS region of the ribosomal operon was proposed by De Jonckheere (2004) for the genus *Naegleria* that was later extended to the Vahlkampfiidae, the most species-rich family within the Heterolobosea (De Jonckheere and Brown 2005). They proposed that unique ITS sequences define species while each genus forms a distinct clade in 5.8S trees. For example, *Naegleria canariensis* was distinguished from *N. gallica* by two single-nucleotide substitutions in the ITS2 sequence (De Jonckheere 2006). Sequences of SSU rDNA, ITS1, 5.8 rDNA, and morphology of the two species are, however, identical. Although many species have been described on the basis of the ITS approach, it could be problematic for several reasons: (1) It is not universal. For example, this concept cannot be used for *Naegleria fowleri* because of its high ITS variability (De Jonckheere 1998, 2004). In addition, little is known about ITS region variability outside the genus *Naegleria*. (2) It was shown that intragenomic polymorphism of the ITS region exists at least in some heteroloboseans. Such taxa could be classified into different species (Dyková et al. 2006). (3) The boundary between intra- and interspecies polymorphism is unknown in most heteroloboseans. This concept assumes the same, almost zero, level of intraspecific variability in all (or almost all) vahlkampfiid species. However, outside the Vahlkampfiidae, e.g. in Psalteriomonadidae or Acrasidae, the molecular polymorphism could be significantly higher, invalidating the concept.

In this study, we decided to identify previously described species and to establish new ones initially on morphological grounds; molecular polymorphisms and phylogeny were used as an accessory criterion. We are aware that our approach, albeit suitable for the Psalteriomonadidae, would be problematic when used for the Vahlkampfiidae as it would considerably underestimate the real number of species. On the other hand, the ITS-based approach clearly overestimates the species number and should be modified to take intraspecies polymorphism into account. Particular species within the Psalteriomonadidae differ in the shape and length of cells, relative flagellar length, nuclear structure, shape of mitochondrial derivates, and lifestyle (freshwater/marine). Our data showed that these characteristics are sufficiently stable among different strains of a single species. Likewise, molecular phylogeny and assessment of SSU rDNA sequence divergence allowed us to identify a flagellated stage of *Monopylocysis visvesvarai* where only the amoebal stage was previously described and allowed confirmation of species identification when we isolated an amoebal-stage (e.g. *P. lanterna*). Our strains belong to at least seven species from five genera, including three new species and two new genera.

Members of the genus *Psalteriomonas* possess a hydrogenosomal complex close to the nucleus. The nucleolus of *Psalteriomonas lanterna* was originally described as central in both flagellate and amoeba stages (Broers et al. 1990). In contrast, we have never seen a centrally located nucleolus in any of our strains of *Psalteriomonas lanterna* and *P. magna* sp. nov. Instead, all the strains possessed one or two parietal nucleoli. Moreover, Prof. J. H. P. Hackstein observed a prominent bright-fluorescing structure within the nucleus, at its periphery, after staining the type strain of *P. lanterna* with ethidium bromide (J. H. P. Hackstein, pers. comm.). This most likely indicates the presence of a parietal nucleolus rather than a central one in the cells of the type strain. If so, the number and position of nucleoli among *P. lanterna* strains seems to be variable.

Psalteriomonas magna sp. nov. differs from *P. lanterna* in the median cell size. With its approximately 70 μm in length, the locomotive amoeba of *P. magna* is about 25 μm longer than the amoeba of *P. lanterna*. We transfer *Lyromonas vulgaris* back to the genus *Psalteriomonas* as originally described by Broers et al. (1993) because of its phylogenetic position and because its cells contain the hydrogenosomal complex (characteristic of the genus). Unfortunately, careful morphological comparison of *P. magna* sp. nov. and *P. vulgaris* comb. nov. is currently not possible as only the amoeba stage is known from the former species and only the flagellated stage is known from the latter. It is worth noting that the amoeba/flagellate length ratio is similar among *Psalteriomonas* species and that the amoeba of *P. magna* sp. nov. is about 40% longer than that of *P. lanterna*, and the *P. vulgaris* flagellate is about 50% smaller than the flagellate of *P. lanterna*. Moreover, *P. magna* amoebae differ from *P. vulgaris* in the presence of individual hydrogenosomes in the cytoplasm. Although we cannot fully exclude the possibility that *P. magna* sp. nov. represents the amoeba stage of *P. vulgaris* comb. nov., we are convinced that they belong to separate species.

The amoebae of our strains of *Sawyeria* are morphologically similar to *Psalteriomonas* strains, but have no hydrogenosomal complex. *Sawyeria marylandensis* is well studied, but the flagellate stage is unknown and is possibly missing altogether. In contrast, at least two species of *Psalteriomonas* are able to form flagellates. One or two parietal

nucleoli located opposite each other in the nucleus were described in the type culture of *S. marylandensis* (O'Kelly et al. 2003). It is consistent with our observation of the strain ITZAVL. However, cells of the strain LUH3 possess more than two parietal nucleoli. We consider this difference to reflect intraspecific variability within *S. marylandensis*, similarly to that of *Psalteriomonas lanterna*.

The genus *Monopylocystis* differs from other psalteriomonadids by a peripheral distribution of nucleolar material (nucleolar ring). Its marine life style is also uncommon in the Psalteriomonadidae, being shared with *Pseudoharpagon pertyi* sp. nov. and an undetermined organism with SSU rDNA sequence AF011464. Only a single species, *M. visvesvarai*, has been described (O'Kelly et al. 2003). We transfer *Vahlkampfia anaerobica* described by Smirnov and Fenchel (1996), which possesses a nucleolar ring and lives in marine environments, to the genus *Monopylocystis*. *Monopylocystis anaerobica* comb. nov. also possesses mitochondrion-related organelles without cristae ("microbody-like structures" in the original description of Smirnov and Fenchel, 1996), a typical feature of the Psalteriomonadidae. *Monopylocystis anaerobica* comb. nov. differs from *M. visvesvarai* in the presence of uroidal filaments and a floating form. The cyst stage has not been found in *M. anaerobica*. Unlike the type cultures of the two species, our *Monopylocystis* strain PC4BIC forms exclusively flagellates under our culturing conditions and neither amoebae nor cysts have been observed. The genetic distance between PC4BIC and the type strain of *M. visvesvarai* was considerably lower than intraspecific molecular polymorphism within related *Harpagon descissus* comb. nov. or *H. schusteri* sp nov. As PC4BIC represents the first case of the flagellate stage in the genus *Monopylocystis* and sequence data of *M. anaerobica* are lacking, at the present time, the problem of species identity of PC4BIC cannot be definitively settled. We provisionally consider the PC4BIC strain to belong to *Monopylocystis visvesvarai* due to the low genetic distance of PC4BIC and the type culture.

Our phylogenetic analysis convincingly showed that *Harpagon descissus* (formerly *Percolomonas descissus*) is unrelated to the presumably closely related *Percolomonas cosmopolitus*. The two species differ considerably in the structure of the mastigont and in their feeding habit. While *H. descissus* feeds by a synchronous action of all four flagella, *P. cosmopolitus* uses the longest flagellum for attaching to the substrate and the remaining three shorter flagella are used to generate a water current for feeding (Fenchel and Patterson 1986). In contrast to *P. cosmopolitus* that possesses discoidal mitochondrial cristae (Fenchel and Patterson 1986), the mitochondrion-related organelle of *H. descissus* is acristate (Brugerolle and Simpson 2004), like in other psalteriomonadids. Therefore, we remove former *P. descissus* from the genus *Percolomonas* and accommodate it in the new genus *Harpagon*. We also describe a second species of the genus *Harpagon*, *H. schusteri* sp. nov. *H. descissus* comb. nov. and *H. schusteri* sp. nov. are morphologically quite similar and the only difference we could detect is the absence of the form with long spike-shaped posterior end in *H. schusteri* sp. nov. This morphologic difference was completely consistent with the molecular phylogenetic analyses that place all isolates of each particular species in a distinct clade that are sister to each other. The genus *Harpagon* gen. nov. contains flagellates with highly polymorphic cells. They differ from members of the genus *Monopylocystis* in the nucleolar structure (several independent parietal nucleoli in *Harpagon* gen. nov., see Brugerolle and Simpson 2004; nucleolar ring in *Monopylocystis*, see O'Kelly et al. 2003, this study), freshwater lifestyle, and shape of the ventral groove.

Pseudoharpagon pertyi gen. nov., sp. nov. is similar to *Harpagon* spp., but differs in several aspects. Its ventral groove is longer and more conspicuous than that of *Harpagon* spp. The cell shape of *P. pertyi* sp. nov. is more stable and cells are about 3 μm shorter than cells of *Harpagon* spp. The two strains of *P. pertyi* sp. nov. were isolated from marine or brackish sediments, whereas members of *Harpagon* are exclusively freshwater. Our data suggest that cells of *Percolomonas descissus* depicted in fig. 3F–J of Bernard et al. (2000) represent, in fact, *P. pertyi* sp. nov.

Phylogeny of the Heterolobosea

The molecular phylogeny of the Heterolobosea is currently inferred almost exclusively from analyses of the SSU rRNA gene (Cavalier-Smith and Nikolaev 2008; De Jonckheere et al. 2009; Nikolaev et al. 2004; O'Kelly et al. 2003; Park and Simpson 2011; Park et al. 2007, 2009; Weekers et al. 1997; Yubuki and Leander 2008). The basal branch of Heterolobosea is in all analyses formed by the sequence designed '*Macropharyngomonas halophila*' which was recently formally described and assigned to the taxon *Pharyngomonas kirbyi* (Park and Simpson 2011). The relationships within the rest of Heterolobosea, Tetramitia *sensu* Cavalier-Smith and Nikolaev (2008), are generally

unresolved in SSU rDNA analyses. Based on the results of previous studies and our analysis, we divide Tetramitia into six robust clades (Fig. 12). To better resolve the phylogeny of Heterolobosea, increased taxon sampling and analyses based on more genes are required. There are still numerous heteroloboseans and organisms tentatively classified within the Heterolobosea that do not have publically available DNA sequence data (e.g. *Gruberella flavescens*, *Percolomonas sulcatus*, *Pernina chaumonti*, *Pseudovahlkampfia emersoni*, *Pocheina* spp., *Tetramastigamoeba hoarei*, *Trimastigamoeba philippinensis*).

Our analysis groups Psalteriomonadidae with genera *Paravahlkampfia*, *Heteramoeba*, *Euplaesiobystra*, *Vrihiamoeba*, *Oramoeba*, and *Stachymoeba* forming together the heterolobosean clade VI. All of these genera except those in Psalteriomonadidae are aerobic suggesting that obligate anaerobiosis appeared relatively recently within the Heterolobosea. Although *Heteramoeba clara, Euplaesiobystra hypersalinica, Stachyamoeba* sp. ATCC 50423 and *Oramoeba fumarolia* are known to form flagellates (De Jonckheere et al. 2011; Droop 1962; Murase et al. 2010; Park et al. 2009), their ultrastructure has yet to be studied in detail, so their relationship with the Psalteriomonadidae cannot be corroborated using morphology alone.

Diversity of the Psalteriomonadidae

The Psalteriomonadidae flourish in sediments with low oxygen concentrations and are found worldwide. *Monopylocystis visvesvarai* and *M. anaerobica* comb. nov. are marine heteroloboseans, *Pseudoharpagon pertyi* sp. nov. was isolated from brackish and marine sediments, and *Psalteriomonas* spp., *Harpagon* spp., and *Sawyeria marylandensis* are inhabitants of freshwater sediments. Cavalier-Smith and Nikolaev (2008) assumed that the immediate ancestor of Psalteriomonadidae was a marine protist and fresh water was invaded only once, by a common ancestor of *Psalteriomonas* and *Sawyeria*. In contrast, our data indicate that marine/freshwater or freshwater/marine transitions were more common than previously hypothesized. At least two such events took place in Psalteriomonadidae. However, available data are not sufficient for pinpointing the directions of these transitions and it is impossible to decide whether the last common ancestor of Psalteriomonadidae was a marine or fresh-water organism. In addition, we showed that environmental sequences FN865111 and FN865530 from an extremely acidic habitat (Amaral-Zettler et al. 2011) represent undescribed psalteriomonadids closely related to the freshwater genera *Psalteriomonas* and *Sawyeria*. Invasions of habitats of high salinity or acidity are not unusual in Heterolobosea and the Psalteriomonadidae is no exception. On the species and generic level, the ecological characteristics seem to be stable in psalteriomonadids.

There is quite a bit of variation in mitochondrial morphology within the Psalteriomonadidae. The mitochondrion of psalteriomonadids does not possess cristae and has been reduced to a hydrogenosome (Barberà et al. 2010; de Graaf et al. 2009). Acristate mitochondrion was also reported from the unrelated species *Pleurostomum flabellatum* (Park et al. 2007), but not in its close relative *Tulamoeba peronaphora* (Park et al. 2009). *Sawyeria marylandensis* has cup-shaped hydrogenosomes that are not surrounded by rough endoplasmic reticulum (Barberà et al. 2010). In *Psalteriomonas vulgaris*, the hydrogenosomes are associated with bacteria and are clustered to form a spherical aggregate situated close to the nucleus (Broers et al. 1993). Interestingly, *P. lanterna* and *P. magna* were reported to possess two hydrogenosomal morphs (Broers et al. 1990; de Graaf et al. 2009; this study). Similar to the two aforementioned species, a huge aggregate of sausage shaped and dumb-bell shaped hydrogenosomes is present in *P. lanterna*. In addition, individual hydrogenosomes surrounded by rough endoplasmic reticulum are also present. The shape of the individual hydrogenosomes is similar to the aggregated ones, or they are bean-shaped. Our TEM study showed that *Monopylocystis visvesvarai* strain PC4BIC possesses a novel morphology of psalteriomonadid mitochondrial derivates. Its mitochondrial derivates are spherical and are not surrounded by rough endoplasmic reticulum. Although mitochondria were not reported in the description of *M. anaerobica* comb. nov. (Smirnov and Fenchel 1996), the 'microbody-like structures' are morphologically identical with the mitochondrion-related organelle in *M. visvesvarai*, except for the diameter, which is smaller in the latter species.

The majority of described heteroloboseans possess a single central nucleolus in the nucleus during interphase (Page and Blanton 1985). In contrast, members of the Psalteriomonadidae possess a number of different nucleolar morphologies. A nucleus with one or a few parietal nucleoli is typical for genera *Psalteriomonas*, *Sawyeria*, and *Harpagon* (Barberà et al. 2010; Brugerolle and Simpson 2004; O'Kelly et al. 2003; this study). The nucleolar material of *Monopylocystis* spp. is

distributed in a thin ring near the nuclear membrane (O'Kelly et al. 2003; Smirnov and Fenchel 1996; this study). The nucleolar morphology of *Pseudoharpagon pertyi* sp. nov. could not be determined.

The mastigont of the psalteriomonadid genera *Psalteriomonas*, *Harpagon* and *Monopylocystis*, is almost identical in fine structure (Broers et al. 1990, 1993; Brugerolle and Simpson 2004; this study). They share the same number and arrangement of flagella, basal bodies, rhizoplasts and their connection to microtubular ribbon, R_1. The ribbon R_1 is the most prominent structure of the mastigont of Psalteriomonadidae and consists of a curved row of thick microtubules originating near the basal body #1. The microtubular fibres that originate in the vicinity of the other three basal bodies consist of only a few microtubules and are not as conspicuous as R_1. One of these poorly developed fibres is an immature R1′ (after Brugerolle and Simpson 2004), which is conspicuous in some other heteroloboseans (e.g. *Tetramitus rostratus*, *Pharyngomonas kirbyi*, and *Percolomonas sulcatus*, see Brugerolle and Simpson 2004; Park and Simpson 2011). The bundle of microfilaments – MB (after Broers et al. 1990; Brugerolle and Simpson 2004) that connects the concave side of R_1 with basal bodies #1 and #4 forms a harp-like structure that represents a synapomorphy of psalteriomonadid flagellates.

The heterolobosean life cycle is variable too. In *Naegleria* and *Willaertia*, the amoeba represents the main trophic stage and the flagellate stage is reduced and does not feed. Flagellates of these two genera are formed under unfavorable conditions and are used for transport (Preston and King 2003). Some authors, however, argued that flagellates of *Naegleria* are gametes (Fulton 1993), but meiosis has been never observed. Flagellates of some heterolobosean genera have not been found at all (e.g. *Vahlkampfia*, *Sawyeria*, *Tulamoeba*, *Neovahlkampfia*, *Paravahlkampfia*). Other heteroloboseans are able to normally feed in both amoeba and flagellate stages (e.g. *Psalteriomonas lanterna*, *Tetramitus* spp.). Finally, representatives of several genera are pure flagellates and the amoeba stage is unknown (e.g. *Stephanopogon*, *Percolomonas*, and *Pleurostomum*). One of the supposed benefits of the phenotypic plasticity of trophozoites is the ability to produce a better phenotype-environment match across more environments than would be possible by producing a single phenotype in all environments. The transformation of the amoeba to flagellate can be induced in vitro in some heterolobosean species (e.g. Fulton 1970; Page 1988; Robinson et al. 1989), while in others attempts were unsuccessful. However, it is highly probable that some heteroloboseans described only as amoebae are able to form flagellates under certain conditions and vice versa. Our inability to induce the transformation in the culture is most likely explained by our limited knowledge of culture demands of many heteroloboseans (e.g. the size, type, and amount of prey in the culture). However, we cannot exclude the possibility that the transformation is connected with meiosis in some species. There is also a possibility that the ability to form flagellates may be blocked by a spontaneous mutation in a gene important for formation of flagella. Importantly, some strains belonging to the genera *Tetramitus*, *Tetramastigamoeba*, *Heteramoeba*, and *Willaertia* were reported to have lost the ability to form the flagellate in the culture (Droop 1962; Page 1988).

Cells of the two strains of *Monopylocystis visvesvarai* appear either as flagellates (strain PC4BIC) or as amoebae and cysts (the type strain) and no sign of the transformation between amoebae and flagellates, or vice versa, has been observed. The strains were cultivated in the same medium. Broers et al. (1990) reported that a minority of *Psalteriomonas lanterna* flagellates could be transformed to amoebae in the culture by an increase in the concentration of oxygen. However, the conversion of the amoeba back to the flagellate was completely unsuccessful. We tried to expose cells of the strain PC4BIC of *Monopylocystis visvesvarai* to oxygen. However, the cells slowly died and no sign of their transformation to amoebae was observed.

The life cycle of the last common ancestor of Psalteriomonadidae was most probably complex and contained three stages: amoeba with eruptive lobopodia, cyst as resting stage, and flagellate with four flagella and ventral feeding groove. The simultaneous presence of two morphological characters can be considered synapomorphies of Psalteriomonadidae: mitochondria without cristae and harp-like structure in the mastigont.

Taxonomic Summary

Family Psalteriomonadidae Cavalier-Smith, 1993

Synonym: Lyromonadidae Cavalier-Smith, 1993.

Diagnosis: Microaerophilic/anaerobic, living in marine, freshwater or brackish sediments. Mitochondrial cristae absent. Ancestrally with amoeba, flagellate, and cyst stage. One or two of the stages lost/unknown in most genera. Flagellates quadriflagellate. Flagellar basal bodies arranged in two lateral pairs, one posterior and one anterior. Basal bodies parallel in a pair. One branching or two associated rhizoplasts, connected to basal bodies #1 and #2. Ribbon R_1 prominent,

R_1' immature. Harp-like structure present in mastigont. Ventral feeding groove present in flagellates. Nucleolus usually not central. Five genera.

Type genus: *Psalteriomonas* Broers, Stumm, Vogels & Brugerolle, 1990.

Other genera: *Monopylocystis* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003; *Sawyeria* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003; *Harpagon* gen. nov.; *Pseudoharpagon* gen. nov.

Remark: Lyromonadidae Cavalier-Smith, 1993 becomes a younger synonym of Psalteriomonadidae Cavalier-Smith, 1993. Although both names were created in the same year, Psalteriomonadidae is older. When Cavalier-Smith created the family Lyromonadidae (Cavalier-Smith 1993b), the name Psalteriomonadidae was already published (Cavalier-Smith 1993a) and was properly cited in Cavalier-Smith (1993b).

Genus *Psalteriomonas* Broers, Stumm, Vogels & Brugerolle, 1990

Diagnosis: Freshwater. Uninucleate to quadrinucleate. Ancestrally with both amoeboid and flagellate stage. Cyst unknown. Cells contain aggregate of hydrogenosomes. Nucleolus parietal, possibly central. Three species.

Type species: *Psalteriomonas lanterna* Broers, Stumm, Vogels & Brugerolle, 1990.

Other species: *P. vulgaris* Broers, Meijers, Symens, Stumm & Vogels, 1993; *P. magna* sp. nov.

Psalteriomonas lanterna Broers, Stumm, Vogels & Brugerolle, 1990

Diagnosis: Both amoeboid and flagellate stage present. Flagellate stage quadrinucleate, with four mastigonts and four feeding grooves. Flagellate ca. 25 μm long. Four equal flagella per mastigont, ca. 1.5 times the cell length. Ventral groove about 2/3 of the cell length. Locomotive amoeba ca. 45 μm long.

Psalteriomonas vulgaris Broers, Meijers, Symens, Stumm & Vogels, 1993

Synonym: *Lyromonas vulgaris* (Broers, Meijers, Symens, Stumm & Vogels, 1993).

Diagnosis: Amoeboid stage unknown. Flagellate stage ca. 12 μm long with single nucleus, mastigont, and ventral groove. Four equal flagella, ca. 1.5 times the cell length. Ventral groove about 2/3 of the cell length.

Psalteriomonas magna sp. nov.

Diagnosis: Flagellate stage unknown. Locomotive amoebae uninucleate, ca. 70 μm long. Long uroidal filaments present in some cells.

Type locality: Kizillar, Turkey. 37°31′N, 35°42′E.

Syntype slides: protargol preparations of the strain KIZILLAR, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 7/40 – 7/43.

Habitat: Freshwater sediments.

Type culture: strain KIZILLAR, deposited in the culture collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Etymology: *magna* [Latin] – great, large. The amoebae of *P. magna* are considerably bigger than that of *P. lanterna*.

Genus *Sawyeria* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: Freshwater. Uninucleate amoebae. Flagellate and cyst stage unknown. Hydrogenosomes individual, cup-shaped, not surrounded by rough endoplasmic reticulum. Nucleus with one to several parietal nucleoli. Single species.

Type species: *Sawyeria marylandensis* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003.

Sawyeria marylandensis O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: As for the genus. Locomotive amoeba ca. 35 μm long.

Genus *Monopylocystis* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: Marine. Uninucleate. Flagellate, cyst and amoeba stage ancestrally present, flagellate and cyst unknown in one species. Nucleolar material peripheral, distributed in a thin ring beneath the nuclear membrane. Cyst possessing single pore plugged with gelatinous material. Two species.

Type species: *Monopylocystis visvesvarai* O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003.

Other species: *M. anaerobica* (Smirnov & Fenchel, 1996) comb. nov.

Monopylocystis visvesvarai O'Kelly, Silberman, Amaral-Zettler, Nerad & Sogin, 2003

Diagnosis: Locomotive amoebae ca. 22 μm long. Floating form unknown. Uroidal filaments absent. Cyst present, with diameter ca. 11 μm. Flagellate stage ca. 16 μm long. Four unequal flagella, one longest, two shorter and equal, one shortest. The longest flagellum up to 1.5 times the cell length. Ventral groove almost reaches the posterior end of the cell.

Monopylocystis anaerobica (Smirnov & Fenchel, 1996) comb. nov.

Synonym: *Vahlkampfia anaerobica* Smirnov & Fenchel, 1996.

Diagnosis: Flagellate unknown. Locomotive amoeba ca. 23 μm long. Uroidal filaments present. Floating form present.

Genus *Harpagon* gen. nov.

Diagnosis: Freshwater. Uninucleate flagellates. Four flagella. Ventral groove up to ½ of the cell length. Amoeba and cyst unknown. Flagellates form several distinct morphotypes. Nucleus with several parietal nucleoli. Two species.

Type species: *Harpagon descissus* (Perty, 1852) comb. nov.

Other species: *Harpagon schusteri* sp. nov.

Etymology: Named after Harpagon, the main character of the Molière's comedy The Miser. The flagellar beating of *Harpagon* spp. and the other members of Psalteriomonadidae is reminiscent of a hand grabbing money.

Harpagon descissus (Perty, 1852) comb. nov.

Synonyms: *Tetramitus descissus* Perty, 1852; *Percolomonas descissus* (Perty, 1852).

Diagnosis: Flagellate ca. 17 μm long. Four unequal flagella, one longest, one shorter, two shortest are equal. Four distinct morphotypes: spindle-shaped cell with a short pointed posterior end, spindle-shaped cell with elongated pointed posterior end, slender elongated cell, pear-shaped short cell with rounded posterior end.

Harpagon schusteri sp. nov.

Diagnosis: Flagellate ca. 17 μm long. Four unequal flagella, one longest, shorter, two shortest are equal. Three distinct morphotypes: spindle-shaped cell with a short pointed posterior end, slender elongated cell, pear-shaped short cell with rounded posterior end. The morphotype with a long pointed posterior end is absent. Differs from *H. descissus* comb. nov. in more than 10% of nucleotides in SSU rDNA.

Type locality: Boroda dam, India. 27°02′N, 76°15′E.

Syntype slides: protargol preparations of the strain INDSIP, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 5/82 – 5/84.

Habitat: Freshwater sediments.

Type culture: strain INDSIP, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Etymology: Dedicated to F. L. Schuster, who passed away in 2009. He was an authority in the field of research and cultivation of pathogenic and opportunistic free-living amoebae. He authored or co-authored many publications on *Naegleria fowleri* and *N. gruberi*.

Pseudoharpagon gen. nov.

Diagnosis: Brackish and marine. Uninucleate flagellates. Ventral groove ½ – 2/3 of the cell length. Amoeba and cyst unknown. Single species.

Type species: *Pseudoharpagon pertyi* sp. nov.

Etymology: *pseudo-* [Greek] – false; in scientific use, denoting close resemblance to the following element. The genus is morphologically similar to *Harpagon* gen. nov.

Pseudoharpagon pertyi sp. nov.

Diagnosis: As for the genus. Flagellate ca. 14 μm long. Four unequal flagella, one longest, three shorter. The longest flagellum up to 1.5 times the cell length.

Type locality: Evros delta, Greece, 40°48′N, 26°01′E.

Habitat: Brackish and marine sediments.

Syntype: Protargol preparations of the isolate EVROS2, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 6/64 and 6/65.

Type culture: EVROS2, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Etymology: Named after outstanding protozoologist M. Perty, who described *Tetramitus descissus*, now *Harpagon descissus*.

Methods

Organisms: The strains were isolated from fresh-water, brackish or marine sediments. Freshwater strains were isolated in Sonneborn's *Paramecium* medium (ATCC medium 802); marine and brackish strains PC4BIC and EVROS2 were isolated in seawater 802 medium (ATCC medium 1525) and marine strain NY0199 was isolated in 5% TYGM-9 medium (ATCC medium 1171) prepared with sterilized seawater. Approximately 2 ml of the samples were initially inoculated into the medium. The cultures were maintained in polyxenic agnothobiotic cultures at room temperature and were subcultured once a week. Most cultures were not monoeukaryotic and contained various other protists besides the heteroloboseans. All strains are available upon request from the collection of the Department of Parasitology, Charles University in Prague, Czech Republic. Monoeukaryotic culture of *Pseudoharpagon pertyi* NY0199 has been deposited at the ATCC, Manassas, VA, USA (accession number PRA-359).

Light microscopy: Light-microscopic observations were performed using an Olympus Microscope BX51 (Olympus Corporation, Tokyo, Japan) equipped with camera Olympus DP70 or a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Oberkochen, Germany) equipped with Leica DC500 digital camera (Leica Microsystems, Wetzlar, Germany). Diameters of

locomotive amoebae and flagellates were measured in 50 cells (30 in NY0199). The morphology of strains EVROS2, INDSIP, ITZAVL, KIZILLAR, PC4BIC, TEXEL, TOCOV, and VIT5 was also examined in protargol-stained preparations. Moist films spread on cover slips were prepared from pelleted cultures obtained by centrifugation at 500 g for 8 minutes. For better adherence to the cover slip, 1 μl of the sample was mixed with 1 μl of egg white diluted to 1:5 with the corresponding cultivation medium prior to the wet smear preparation. The films were then fixed in Bouin-Hollande's fluid for ca. 5 hours, were washed with 70% ethanol, and were stained with 1% protargol (Bayer, I. G. Farbenindustrie Actinengesellschaft) following the Nie's (1950) protocol.

Transmission electron microscopy: Cells of strains PC4BIC and KIZILLAR were pelleted by centrifugation at 500 g for 8 minutes, resuspended in a solution containing 2.5% glutaraldehyde (Polysciences) and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.2), and fixed at room temperature for 4 hours. After washing in 0.1 M cacodylate buffer (three times per 15 minutes), the cells were postfixed with 2% OsO_4 in 0.1 M cacodylate buffer for 3 hours. After washing with an excess volume of 0.1 M cacodylate buffer (three times per 15 minutes) the fixed cells were dehydrated in an acetone series and embedded in Epon resin (Poly/Bed 812, Polysciences). The ultrathin sections were stained with lead citrate and uranyl acetate (2 – 3%) and examined using a TEM JEOL 1011 transmission electron microscope.

DNA isolation, amplification, cloning and sequencing: Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen), PureGen Tissue DNA isolate kit (Qiagen), or MasterPure Complete DNA and RNA purification Kit (Epicentre, WI, USA) according to the manufacturer's instructions. Almost complete SSU rDNA of most strains was amplified using primers MedlinA (CTGGTTGATCCTGCCAG) and MedlinB (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al. 1988) with an annealing temperature of 50 °C, except for *H. descissus* strains BAUM and WS, which utilized 42 °C. SSU rDNA of the strain NY0199 was amplified using NPF1 (TGCGCTACCTGGTTGATCC) and R4 (GATCCTTCTGCAGGTTCACCTAC) with an annealing temperature of 49 °C. SSU rDNA of strains CERETE, CIZOV1, IND7, KIZILLAR, LUH2, LUH3, VIT5, and VIT9 was amplified using primers HETERF1 (GCTTATTTCRAAGATTAAGCCATGYAAA) and HETER1K (AAYTCAGGGACGTAATCATT), which were newly designed on the basis of available SSU rDNA sequences of freshwater Psalteriomonadidae (genera *Psalteriomonas*, *Sawyeria*, and *Harpagon*), with an annealing temperature of 60 °C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), Zymoclean™ GEL DNA Recovery Kit (Zymo Research) or UltraClean 15 DNA Purification Kit (MO BIO) and were either directly sequenced or were cloned into either the TOPO T/A vector pCR4 or the pGEM®-T EASY vector using the pGEM®-T EASY VECTOR SYSTEM I (Promega) and at least two clones were sequenced. Sequence data reported in this paper are available in GenBank under accession numbers JN606327 – JN606357.

Phylogenetic analyses: A data sets containing 31 newly determined SSU rDNA sequences, 48 sequences of Heterolobosea retrieved from GenBank, and 9 sequences of other Discoba (Jakobida, Euglenozoa, *Tsukubamonas*, 'Soginia') used as outgroups, was created. The sequences were aligned using the MAFFT method (Katoh et al. 2002) with the help of the MAFFT 6 server http://mafft.cbrc.jp/alignment/server/ with the G-INS-i algorithm at default settings. The alignment was manually edited in BioEdit 7.0.4.1 (Hall 1999). The final data set contained 1246 aligned characters and is available from the corresponding author upon request. Phylogenetic trees were constructed by maximum likelihood and Bayesian methods. Maximum likelihood analysis was performed in RAxML 7.0.3 (Stamatakis 2006) under the GTRGAMMAI model. Bootstrap support values were generated in RAxML from 1000 pseudoreplicate data sets. Bayesian analysis was performed using MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003) under the GTR + I + Γ + covarion model. Four MCMCs were run for $4{\cdot}10^6$ generations, until the mean standard deviation of split frequencies based on last 75% generations was lower than 0.01. The trees were sampled every 100th generation. The first 25% of trees were removed as burn-in.

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5.2. Pánek *et al.* 2014a

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Survey on diversity of marine/saline anaerobic Heterolobosea (Excavata: Discoba) with description of seven new species

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Diversity of the anaerobic Heterolobosea (Excavata: Discoba) is only poorly understood, especially in marine environments. We have isolated and cultured 16 strains of anaerobic heteroloboseid amoebae and flagellates from brackish, marine and saline anoxic habitats worldwide. Phylogenetic analyses of SSU rDNA sequences and light-microscopic observations showed that all the strains belong to the family Psalteriomonadidae, the main anaerobic lineage of Heterolobosea, and that they represent eight species from the genera *Monopylocystis*, *Harpagon* and *Pseudoharpagon.* Seven species are newly isolated and described here as *Monopylocystis minor* n. sp., *Monopylocystis robusta* n. sp., *Monopylocystis elegans* n. sp., *Monopylocystis disparata* n. sp., *Harpagon salinus* n. sp., *Pseudoharpagon longus* n. sp. and *Pseudoharpagon tertius* n. sp. Amoebae, cysts and the ultrastructure of the genus *Pseudoharpagon* are presented for the first time.

Introduction

Heterolobosea is a distinctive lineage of protists belonging to the eukaryotic supergroup Excavata (Adl *et al.*, 2005). The group was coined by Page & Blanton (1985) to acknowledge that shizopyrenid limax amoebae/amoeboflagellates and acrasid slime moulds together constitute a monophyletic group. Currently, the taxon Heterolobosea encompasses approximately 140 described species (Pánek & Čepička, 2012) and is divided into the subphyla Tetramitia, which comprises the vast majority of the species, and Pharyngomonada, with only two species of the genus *Pharyngomonas* (Cavalier-Smith & Nikolaev, 2008; Harding *et al.*, 2013). A potentially separate evolutionary lineage of the Heterolobosea, represented by strain BB2, was discovered recently (Harding *et al.*, 2013). Many heteroloboseids are true amoeboflagellates, i.e. they switch between an aflagellate amoeba and a flagellate, and vice versa, during the life cycle. Interestingly, the amoebae entirely lack cytoplasmic microtubules. The flagellar apparatus, including basal bodies, is formed *de novo* during the amoeba–flagellate transformation (Walsh, 2007; Lee, 2010). Several heteroloboseid species have secondarily lost one of the two motile stages and exist as pure amoebae (e.g. species of the genera *Vahlkampfia*, *Neovahlkampfia* and *Paravahlkampfia*) or flagellates (e.g. *Percolomonas cosmopolitus*, species of the genera *Harpagon* and *Stephanopogon*) (see Pánek & Čepička, 2012). In addition, many heteroloboseid species form resistant cysts in harsh conditions.

Heteroloboseid amoebae are usually cylindrical and move with eruptive lobopodia. Amoebae of individual species or even genera are often morphologically virtually indistinguishable, and their morphology cannot be used in taxonomical studies (see e.g. Brown & De Jonckheere, 1999). By contrast, the flagellates are much more variable in their external morphology. They are almost always tetrakont or bikont (i.e. equipped with four or two flagella). A higher number of flagella is known from only a few heteroloboseids: (1) Flagellates of *Psalteriomonas lanterna*, but not that of *Psalteriomonas vulgaris*, possess four ventral grooves, four nuclei and four tetrakont mastigonts (Broers *et al.*, 1990); (2) members of the genus *Stephanopogon* are obligate flagellates with several tens of flagella and ciliate-like gross morphology (Yubuki & Leander, 2008); (3) flagellates of the recently discovered *Oramoeba fumarolia* were described to possess two to ten flagella, but their morphology has not been studied in detail (De Jonckheere *et al.*, 2011a). Phylogenetic analyses

Abbreviations: BPP, Bayesian posterior probability; BS, bootstrap support; DIC, differential interference contrast; MRO, mitochondrion-related organelle.

The GenBank/EMBL/DDBJ accession numbers for newly determined SSU rDNA sequences are KF840524–KF840527 and KF840534 (*Monopylocystis visvesvarai*), KF840522, KF840523, and KF840528 (*Monopylocystis disparata* n. sp.), KF840533 (*Monopylocystis robusta* n. sp.), KF840521 and KF840529 (*Monopylocystis minor* n. sp.), KF840536 (*Monopylocystis elegans* n. sp.), KF840530 (*Pseudoharpagon longus* n. sp.), KF840531 and KF840532 (*Pseudoharpagon tertius* n. sp.) and KF840535 (*Harpagon salinus* n. sp.).

Eight supplementary figures are available with the online version of this paper.

of SSU rDNA showed that these three lineages are not specifically related (De Jonckheere *et al.*, 2011a). The heterolobosеid flagellates usually possess a conspicuous ventral groove. The groove is considered to be homologous to that of other excavates, although it is supported by partially different cytoskeletal elements (Brugerolle & Simpson, 2004; Park & Simpson, 2011). The groove of some species, e.g. those of the genus *Naegleria*, has been lost, and the flagellates are short-lived and transient (Fulton, 1993; De Jonckheere, 2002).

Although heterolobosеids are not a species-rich group, they are extremely diverse in their ecology. In particular, a significant number of species have been detected in various extreme habitats, such as thermal springs, Antarctic lakes, and hypersaline or highly acidic habitats (Amaral-Zettler *et al.*, 2002, 2011; Murtagh *et al.*, 2002; Garstecki *et al.*, 2005; Park *et al.*, 2007, 2009; Baumgartner *et al.*, 2009; De Jonckheere *et al.*, 2009, 2011a, b; Park & Simpson, 2011). Ten species of obligately anaerobic/microaerophilic heterolobosеids have been described so far (Perty, 1852; Ruinen, 1938; Broers *et al.*, 1990, 1993; Smirnov & Fenchel, 1996; O'Kelly *et al.*, 2003; Brugerolle & Simpson, 2004; Pánek *et al.*, 2012). It was recently shown that all but one of them belong to a single clade, the Psalteriomonadidae (Pánek *et al.*, 2012). A separate lineage of presumably anaerobic heterolobosеids is represented by the halophilic species *Pleurostomum flabellatum* (Park *et al.*, 2007). However, its anaerobiosis has yet to be confirmed. The nine species of the family Psalteriomonadidae are currently grouped into five genera: *Psalteriomonas*, *Sawyeria*, *Monopylocystis*, *Harpagon* and *Pseudoharpagon*. All known members of the Psalteriomonadidae possess mitochondrion-related organelles lacking cristae (de Graaf *et al.*, 2009; Barberà *et al.*, 2010; Pánek *et al.*, 2012) and a characteristic mastigont structure of the flagellates (Pánek *et al.*, 2012). The ancestral life cycle (with separate amoeba and flagellate stages) has been retained in some species, such as *Psalteriomonas lanterna* and *Monopylocystis visvesvarai*, but most species seem to have permanently lost one of the stages and are known exclusively as amoebae (e.g. *Sawyeria marylandensis*) or flagellates (e.g. species of the genus *Harpagon*, *Pseudoharpagon pertyi*). *Monopylocystis visvesvarai* is the only member of the family Psalteriomonadidae known to be able to produce cysts (O'Kelly *et al.*, 2003). The cysts are unique among the Heterolobosea by having a single pore occluded by a gelatinous plug, hence the name *Monopylocystis* (literally 'cyst with a single pore').

Members of the family Psalteriomonadidae are predominantly freshwater, and only three species have been found in marine or brackish anoxic sediments: *M. visvesvarai*, *Monopylocystis anaerobica* (formerly *Vahlkampfia anaerobica*) and *Pseudoharpagon pertyi*. Moreover, each of them is represented by only one or two strains. To assess the diversity of anaerobic heterolobosеids in brackish/marine habitats and inland salt marshes, we isolated and cultured 16 strains from various localities worldwide. According to light-microscopic morphology and SSU rDNA sequence analysis, our strains are unexpectedly diverse and represent eight distinct species of the family Psalteriomonadidae, seven of which are novel. We also present the ultrastructure of the genus *Pseudoharpagon* for the first time.

Methods

Organisms. Information on the origin of strains included in the study is summarized in Table 1. Most strains were isolated between 2009 and 2011 from marine or brackish coastal sediments. Strains TSUKIM (*Harpagon salinus* n. sp.) and TSUKIMV (*M. visvesvarai*) were obtained in 2012 from an inland salt marsh. Strains FUEN3 (*Monopylocystis robusta* n. sp.) and FUEN4 (*Monopylocystis disparata* n. sp.) were isolated in 2011 from saline sediments collected near a salt pan. Strain COORONG of *M. disparata* n. sp. was isolated in 2010 from a similar environment. All strains were isolated in the seawater-based ATCC medium 1525; approximately 2 ml of the samples was initially inoculated into the medium. The strains were maintained in polyxenic agnothobiotic cultures with unidentified bacteria at room temperature, and they were subcultured once a week. The cultures, except for LAGOS1P (*Pseudoharpagon tertius* n. sp.), were not monoeukaryotic and contained various other protists besides the heterolobosеids. Strains LAGOS1M (*M. disparata* n. sp.) and LAGOS1P (*Pseudoharpagon tertius* n. sp.), and strains EVROS1M (*Monopylocystis elegans* n. sp.) and EVROS1I (*Pseudoharpagon longus* n. sp.), respectively, were originally isolated from a single sample, but were successfully separated and cultured individually after several initial passages. Strains TSUKIMV (*M. visvesvarai*) and TSUKIM (*H. salinus* n. sp.) were isolated from a single sample that was inoculated into two tubes containing ATCC medium 1525, and two separate cultures, A and B, were created. Culture A contained a single heterolobosean, *M. visvesvarai*, whereas culture B contained members of both the genera *Monopylocystis* and *Harpagon*. Culture B was later transferred into the freshwater-based ATCC medium 802 in order to examine the ability of *H. salinus* n. sp. to grow in freshwater environments.

Light microscopy. Light-microscopic observations were performed using an Olympus Microscope BX51 equipped with an Olympus DP71 camera. Diameters of locomotive amoebae and flagellates were measured in 30 cells. In addition, the morphology of eight strains was examined in protargol-stained preparations. Moist films spread on coverslips were prepared from pelleted cultures obtained by centrifugation at 500 ***g*** for 8 min. For better adherence to the coverslip, 1 µl of the sample was mixed with 1 µl egg white diluted to 1 : 4 with the corresponding cultivation medium prior to the wet smear preparation. The films were then fixed in Bouin-Hollande's fluid: cupric sulfate 2.5 g, picric acid 4 g, distilled water 100 mL, 40 % formaldehyde 10 mL, acetic acid 1.5 mL. for 5 h, were washed with 70 % ethanol, and were stained with 1 % protargol (Bayer) following the protocol of Nie (1950).

Transmission electron microscopy. The cells of *Pseudoharpagon pertyi* strain EVROS2N and *Pseudoharpagon tertius* n. sp. strain LAGOS1P were pelleted by centrifugation at 2300 ***g*** for 5 min and fixed for 30 min at room temperature in 2 ml of a cocktail containing growth medium and 0.16 ml 25 % (v/v) glutaraldehyde (final concentration 2 %). The cells were rinsed three times with the growth medium and were post-fixed for 1 h in 1 ml of a cocktail containing 0.25 ml 4 % (w/v) OsO_4 (final concentration 1 %) and 0.75 ml of growth medium. After being rinsed free of post-fixative, the cells were concentrated by centrifugation and were trapped in 2 % (w/v) agarose. The fixed cells were dehydrated in an acetone series and embedded in Epon resin (Poly/Bed 812/Araldite 502, Polysciences). The ultrathin sections were cut using a diamond knife on an Ultracut

Table 1. List of newly isolated strains

B, brackish coastal sediments; I, inland salt marsh sediments; M, marine coastal sediments; S, saline coastal sediments.

Species	Strain	Habitat	Locality	Coordinates
Monopylocystis visvesvarai	GOUVIA	B	Gouvia, Corfu Island, Greece	39° 38′ N 19° 50′ E
	IGO2	B	Igoumenitsa, Greece	39° 31′ N 20° 14′ E
	IGO3	B	Igoumenitsa, Greece	39° 31′ N 20° 14′ E
	KORISSION3	M	Lake Korission, Korfu, Greece	39° 27′ N 19° 52′ E
	TSUKIMV	I	Einot Tsukim Nature Reserve, Israel	31° 42′ N 35° 27′ E
Monopylocystis disparata n. sp.	COORONG	S	Coorong NP, Australia	36° 04′ S 139° 35′ E
	FUEN4	S	Fuencaliente, La Palma island, Spain	28° 27′ N 17° 50′ W
	LAGOS1M	M	Porto Lagos, Greece	41° 00′ N 25° 06′ E
Monopylocystis minor n. sp.	AND	M	Elephant beach, Havelock Island, India	12° 00′ N 92° 56′ E
	TINI	M	Tiwi, Oman	22° 49′ N 59° 15′ E
Monopylocystis elegans n. sp.	EVROS1M	B	Evros delta, Greece	40° 48′ N 26° 01′ E
Monopylocystis robusta n. sp.	FUEN3	S	Fuencaliente, La Palma island, Spain	28° 27′ N 17° 50′ W
Pseudoharpagon longus n. sp.	EVROS1I	B	Evros delta, Greece	40° 48′ N 26° 01′ E
Pseudoharpagon tertius n. sp.	LAGOS1P	M	Porto Lago, Greece	41° 00′ N 25° 06′ E
	MURANO3	M	Murano Islands, Venetian Lagoon, Italy	45° 27′ N 12° 21′ E
Harpagon salinus n. sp.	TSUKIM	I	Einot Tsukim Nature Reserve, Israel	31° 42′ N 35° 27′ E

E ultramicrotome (Reichert), stained with lead citrate and saturated uranyl acetate, and examined using a JEM 1011 (JEOL) transmission electron microscope.

DNA isolation, amplification, cloning and sequencing. Genomic DNA was isolated using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. The almost complete SSU rRNA gene of strains KORISSION3 (*M. visvesvarai*), LAGOS1P (*Pseudoharpagon tertius* n. sp.), LAGOS1M (*M. disparata* n. sp.), TSUKIM (*H. salinus* n. sp.) and TSUKIMV (*Monopylocystis visvesvarai*) was amplified using primers MedlinA (5′-CTGGTTGATCCTGCCAG-3′) and MedlinB (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin *et al.*, 1988) with an annealing temperature of 55 °C. The SSU rRNA gene of the remaining strains was amplified using primers HETANF (5′-GYTTATYTCRARGATTAAGCCATGYAAA-3′) and HETANR (5′-AAYTCAGKGACGTADTCADT-3′) with an annealing temperature of 55 °C. The primers were derived from primers HETERF1 and HETER1K (Pánek *et al.*, 2012) in order to cover members of the genera *Monopylocystis* and *Pseudoharpagon*. PCR products were purified using a QIAquick PCR Purification kit (Qiagen) or Zymoclean GEL DNA Recovery kit (Zymo Research) and were usually directly sequenced. The SSU rRNA gene of the strains TSUKIM (*H. salinus* n. sp.), TSUKIMV (*M. visvesvarai*) and LAGOS1M (*M. disparata* n. sp.) was cloned into the pGEM-T EASY vector using the pGEM-T EASY VECTOR SYSTEM I (Promega), and two to eight clones were sequenced.

Phylogenetic analyses. Two datasets consistinsg of SSU rDNA sequences were created. The first dataset contained 16 newly determined sequences and 77 sequences representing major lineages of Heterolobosea retrieved from GenBank. The second dataset contained 16 newly determined sequences and 12 sequences of members of the family Psalteriomonadidae retrieved from GenBank. The sequences were aligned using the MAFFT method (Katoh *et al.*, 2002) with the help of the mafft 7 server (http://mafft.cbrc.jp/alignment/server/) with the G-INS-i algorithm at default settings. The alignment was manually edited in BioEdit 7.0.4.1 (Hall, 1999). The final datasets contained 1208 and 1768 aligned characters, respectively, and are available from the corresponding author upon request. Phylogenetic trees were reconstructed by maximum-likelihood and Bayesian methods. Maximum-likelihood analysis was performed in RAxML 7.0.3 (Stamatakis, 2006) under the GTRGAMMAI model. Bootstrap support values were generated in RAxML from 1000 pseudoreplicate datasets. Bayesian analysis was performed using MrBayes 3.2 (Ronquist *et al.*, 2012) under the GTR + I + Γ + covarion model. Four Markov chain Monte Carlo (MCMC) chains were run for 5.10^6 and 1.10^6 generations, respectively, until the mean standard deviation of split frequencies based on last 75 % generations was lower than 0.01. The trees were sampled every 500th generation. The first 25 % of trees were removed as burn-in.

Results

Light-microscopic observations

Uninucleate flagellates, amoebae and cysts were observed in the cultures. The cells were anaerobic/microaerophilic and were always found at the bottom of the culture tubes. Cell diameters of particular stages are summarized in Table 2. The morphology of particular life stages of the studied strains is shown in Figs 1–6; Figs S1–S8 (available in the online Supplementary Material) summarize the morphology of each species.

General morphology of species of the genus *Monopylocystis*. Twelve strains were classified into the genus *Monopylocystis*. The amoebae of species of the genus *Monopylocystis* displayed characteristic features of Heterolobosea; they were monopodial in locomotion and possessed an anterior eruptive hyaline front reaching approximately from 1/5 to 1/3 of the cell length (Fig. 1a–s). The anterior hyaloplasm was clearly separated from the posterior granuloplasm. Floating amoeboid cells of all strains were spherical and did not form pseudopodia (not shown). The nucleus was usually situated in the anterior part of the granuloplasm. It was rounded in some cells, but often strongly deformed during the locomotion. The

Table 2. Dimensions of living specimens of heterolobosean strains

Mean ± SD of 30 specimens (smallest–largest value). A, amoeba; F, flagellate; NA, not available.

Species	Strain	Stage	Cell length	Cell width	LW ratio	Cyst diameter
Monopylocystis visvesvarai	GOUVIA	A	20.6 ± 2.9 (15.7 – 25.3)	5.7 ± 1.0 (4.1 – 8.2)	3.7 ± 0.6 (2.6 – 4.6)	n.a.
	IGO2	A	27.9 ± 5.8 (16.0 – 42.2)	10.1 ± 2.6 (5.3 – 17.3)	2.9 ± 0.7 (1.6 – 4.7)	n.a.
	IGO3	A	27.6 ± 5.1 (18.4 – 37.3)	9.6 ± 1.0 (8.2 – 11.1)	2.9 ± 0.7 (1.9 – 5.1)	11.4 ± 1.1 (9.0 – 13.4)
	KORISSION3	A	24.8 ± 2.6 (19.3 – 28.4)	7.6 ± 1.7 (6.0 – 9.7)	3.4 ± 0.5 (1.9 – 4.2)	11.6 ± 1.2 (8.1 – 14.0)
		F	16.4 ± 1.3 (13.9 – 18.6)	n.a.	n.a.	NA
	TSUKIMV	A	24.1 ± 5.5 (17.7 – 39.7)	9.9 ± 1.7 (6.4 – 13.1)	2.5 ± 0.6 (1.6 – 4.7)	9.8 ± 0.5 (8.4 – 10.7)
Monopylocystis disparate n. sp.	FUEN4	A	16.4 ± 3.8 (9.3 – 31.0)	6.4 ± 1.4 (4.2 – 11.4)	2.6 ± 0.4 (1.8 – 3.5)	7.8 ± 0.9 (6.6 – 9.8)
	LAGOS1M	F	24.2 ± 2.2 (19.6 – 28.9)	n.a.	n.a.	n.a.
Monopylocystis minor n. sp.	AND	A	18.2 ± 5.0 (13.0 – 31.0)	5.9 ± 0.8 (4.0 – 7.0)	3.1 ± 0.8 (2.2 – 5.2)	8.7 ± 1.2 (7.2 – 11.8)
		F	11.0 ± 1.0 (10.7 – 13.5)	n.a.	n.a.	NA
	TINI	A	16.6 ± 3.4 (10.0 – 22.0)	4.9 ± 1.0 (3.0 – 7.0)	3.4 ± 0.6 (2.6 – 4.5)	8.7 ± 1.0 (7.3 – 11.4)
Monopylocystis elegans n. sp.	EVROS1M	A	23.2 ± 5.5 (16.0 – 50.6)	8.0 ± 1.6 (4.0 – 12.3)	3.0 ± 0.6 (1.9 – 5.0)	9.0 ± 1.0 (7.4 – 11.1)
		F	18.1 ± 3.1 (12.9 – 25.3)	n.a.	n.a.	NA
Monopylocystis robusta n. sp.	FUEN3	A	32.2 ± 8.1 (18.4 – 50.8)	11.2 ± 2.6 (5.7 – 16.9)	2.9 ± 0.6 (1.8 – 4.6)	12.2 ± 1.2 (9.7 – 13.9)
Pseudoharpagon longus n. sp.	EVROS1I	A	38.5 ± 6.8 (29.3 – 56.1)	12.6 ± 2.7 (8.6 – 19.4)	3.1 ± 0.5 (2.1 – 3.8)	19.0 ± 1.4 (16.5 – 22.4)
		F	20.4 ± 4.1 (14.0 – 34.8)	n.a.	n.a.	NA
Pseudoharpagon tertius n. sp.	LAGOS1P	F	15.5 ± 3.3 (10.5 – 22.0)	n.a.	n.a.	n.a.
Harpagon salinus n. sp.	TSUKIM	F	13.7 ± 1.4 (11.2 – 17.0)	n.a.	n.a.	n.a.

nucleus of amoebae of all strains possessed a thin peripheral layer of nucleolar material and homogeneous central area. The peripheral layer was not of uniform thickness and displayed several granules protruding to the karyoplasm. In protargol-stained cells, the nuclear structure appeared more complex: beneath the peripheral layer, which was well stained, there was a large, heavily stained globule. The globule was separated from the peripheral layer by an unstained zone (Fig. 2a–n). The amoebae of particular strains were morphologically similar and differed in cell diameters, form of uroid and uroidal filaments, and position of the nucleus.

The flagellates were quadriflagellate and possessed a conspicuous ventral groove (Fig. 3); the flagella inserted anterolaterally, were parallel and were not associated with the ventral groove. The ventral groove reached almost the posterior end of the cell. Similarly to *Monopylocystis* amoebae, the nucleus of flagellates of all species possessed a thin peripheral layer of nucleolar material (Fig. 3a, e, h, o). Flagellates of particular species differed in cell diameters.

Cysts were present in many cultures of species of the genus *Monopylocystis*, where they co-occurred with amoebae. Cysts of species of the genus *Monopylocystis* were similar to cysts of *M. visvesvarai* described by O'Kelly *et al.* (2003), i.e. they possessed a single prominent pore occluded by a plug (Fig. 6a–h). The cytoplasm within cysts contained many tiny globules, and sometimes a single nucleus was observed (Fig. 6h).

***Monopylocystis visvesvarai*.** Amoebae were observed in the cultures of all five strains of *M. visvesvarai*: GOUVIA, IGO2, IGO3, KORISSION3 and TSUKIMV (Fig. 1a–g).

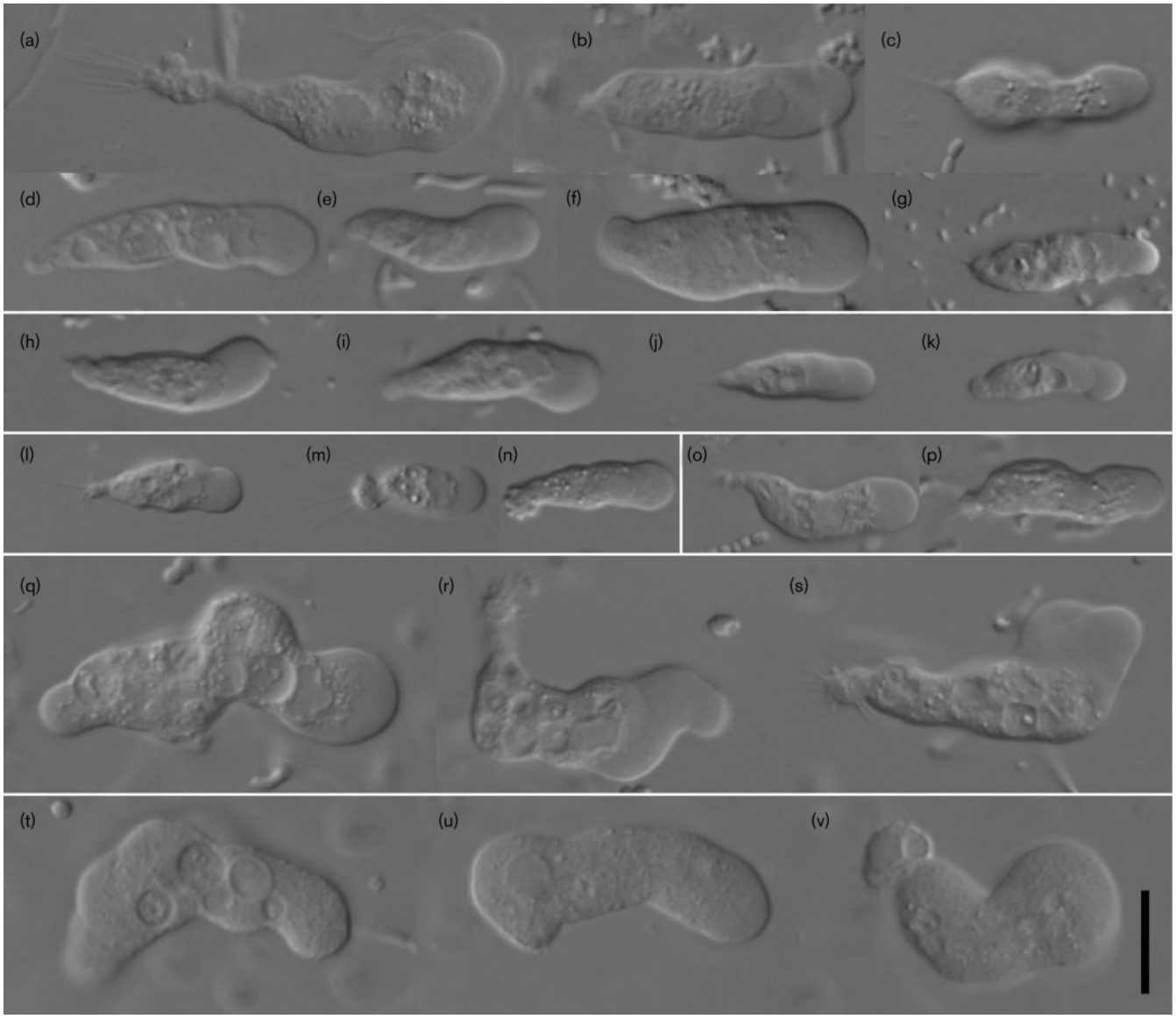

Fig. 1. Locomotive amoebae of *M. visvesvarai* strains IGO2 (a, b), KORISSION3 (c, d), GOUVIA (e), IGO3 (f) and TSUKIMV (g), *Monopylocystis minor* n. sp. strains AND (h, i) and TINI (j, k), *M. disparata* n. sp. strain FUEN4 (l–n), *M. elegans* n. sp. strain EVROS1M (o, p), *M. robusta* n. sp. strain FUEN3 (q–s) and *Pseudoharpagon longus* n. sp. strain EVROS1I (t–v). Differential interference contrast (DIC). Bar, 10 μm.

The amoebae were variable in size, and the mean length of locomotive specimens differed by 7 μm between strain GOUVIA with the smallest cells and strain IGO2 with the largest cells (Table 2). The nucleus was located at the anterior end of the granuloplasm during rapid locomotion. Most locomotive cells formed a bulbous or villous-bulbous uroid, which occasionally bore trailing filaments (Fig. 1a, c, g). Flagellates were observed only in the culture of strain KORISSION3 (Figs. 3a–c); they were morphologically identical to flagellates of strain PC4BIC described by Pánek *et al.* (2012). Cysts with typical morphology of *Monopylocystis* were present in the culture of strains IGO3, KORISSION3 and TSUKIMV (Fig. 6a–c).

***Monopylocystis minor* n. sp.** Both strains of *M. minor* n. sp., AND and TINI, consisted of amoebae, cysts and flagellates in early passages of the culture; the flagellates of strain TINI, however, disappeared after a few tens of passages. The amoebae of *M. minor* n. sp. (Fig. 1h–k) were slightly smaller than those of *M. visvesvarai* (Table 2). The nucleus was located at the anterior end of the granuloplasm in locomotive amoebae and was always strongly deformed. The cells usually formed a bulbous uroid, sometimes with a single short filament (Fig. 1h, j). Flagellates of both strains were smaller than flagellates of *M. visvesvarai* (Figs 3d–g, 5a, b). The cysts displayed typical morphology of the genus *Monopylocystis* (Fig. 6d, e).

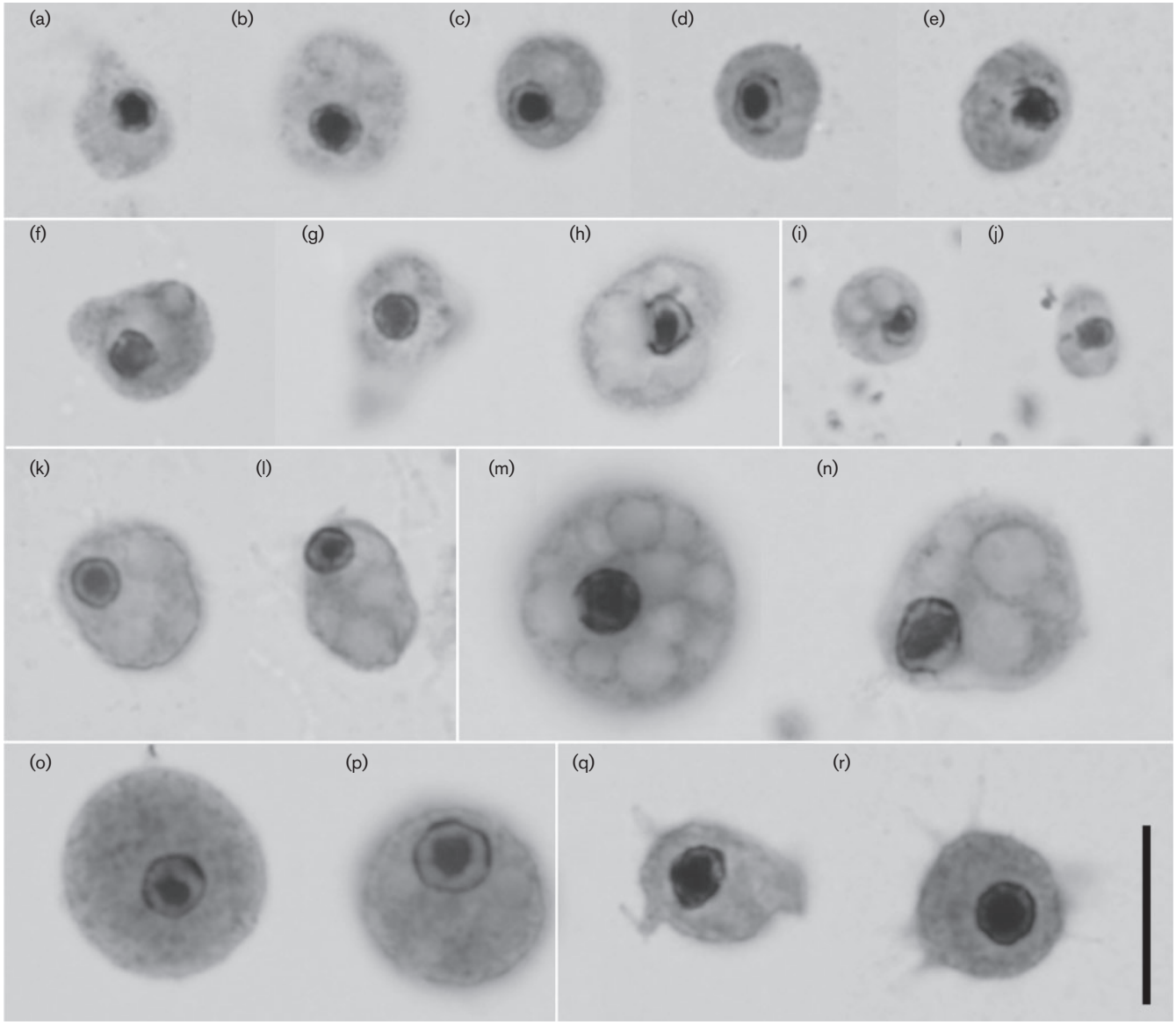

Fig. 2. Protargol-stained amoebae of *M. visvesvarai* strains IGO2 (a, b), KORISSION3 (c, d) and GOUVIA (e), *M. minor* n. sp. strains AND (f, g) and TINI (h), *M. disparata* n. sp. strain FUEN4 (i, j), *M. elegans* n. sp. strain EVROS1M (k, l), *M. robusta* n. sp. strain FUEN3 (m, n), *Pseudoharpagon longus* n. sp. strain EVROS1I (o, p) and *Pseudoharpagon tertius* n. sp. strain LAGOS1P (q, r). Bright field. Bar,10 μm.

***Monopylocystis disparata* n. sp.** Amoebae and cysts were observed in the culture of strain FUEN4; only flagellates were observed in the culture of strain LAGOS1M. Strain COORONG was lost before its morphology could be examined. The amoebae of strain FUEN4 were small in comparison with those of the other *Monopylocystis* strains (Fig. 1l–n, Table 2). The nucleus of locomotive amoebae was located at the anterior end of the granuloplasm. Most cells possessed a well-differentiated villous-bulbous uroid, often with several long filaments. Cysts of strain FUEN4 were smaller than the cysts of the other *Monopylocystis* strains and displayed typical morphology of the genus (Fig. 6f). Flagellates of *M. disparata* n. sp. strain LAGOS1M were the largest among members of the genus *Monopylocystis* (Fig. 3h–k, Table 2).

***Monopylocystis elegans* n. sp.** Amoebae, flagellates and cysts were observed in the culture of strain EVROS1M. The nucleus of locomotive amoebae was located deeper in the granuloplasm and was not deformed during the locomotion (Fig. 1o, p); in protargol-stained cells, the shape of the nucleus was more regular than in cells of the other species (Fig. 2k, l). The cells usually formed a uroid, sometimes with short filaments. Flagellates of strain EVROS1M were

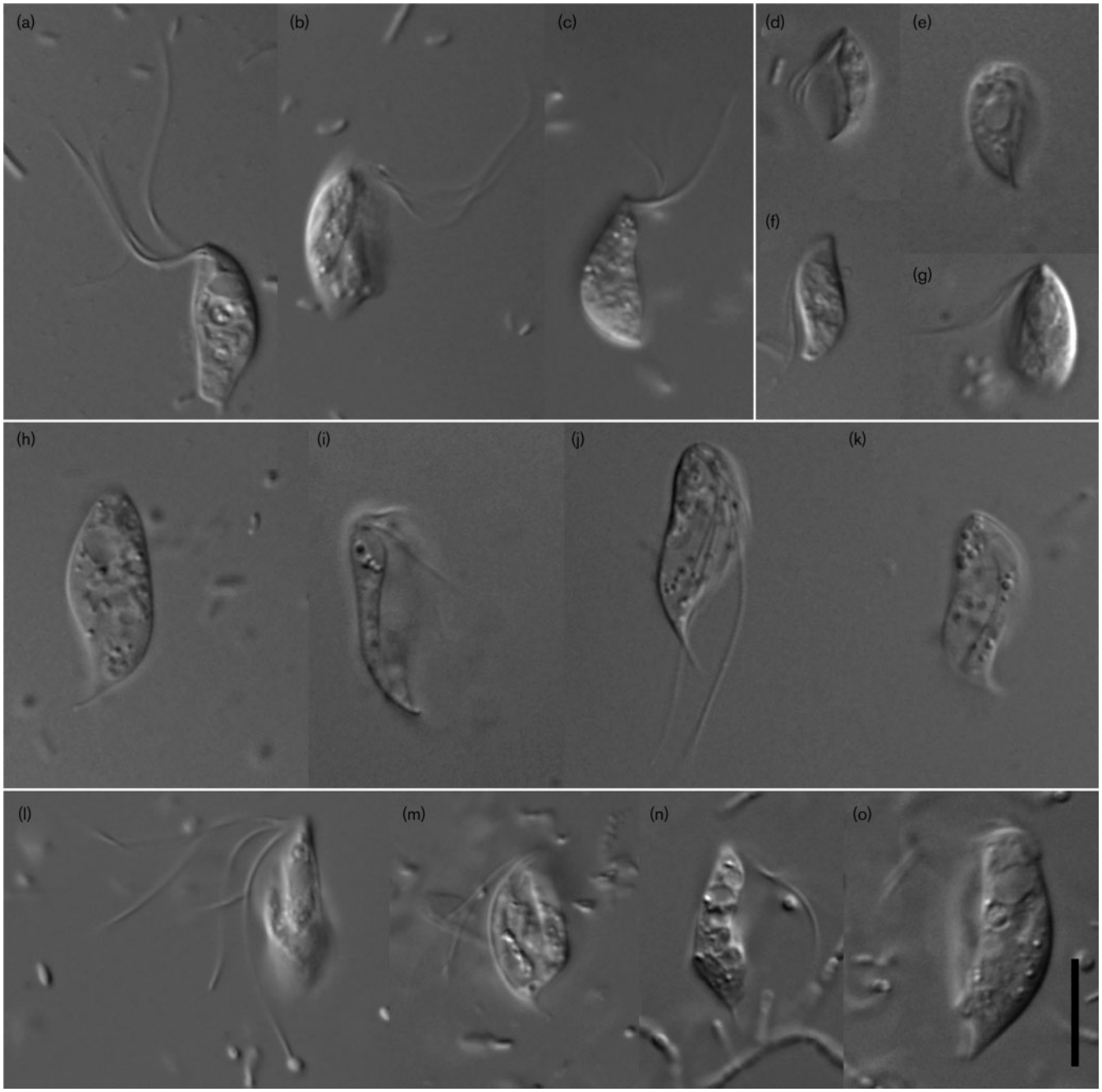

Fig. 3. Living flagellates of *M. visvesvarai* strain KORISSION3 (a–c), *M. minor* n. sp. strains AND (d–f) and TINI (g), *M. disparata* n. sp. strain LAGOS1M (h–k) and *M. elegans* n. sp. strain EVROS1M (l–o). DIC. Bar, 10 μm.

similar to that of *M. visvesvarai* (Figs 3l–o, 5c, d); typically, they were slightly larger and more variable in length (Table 2). The cysts displayed typical morphology of *Monopylocystis* (Fig. 6g).

***Monopylocystis robusta* n. sp.** Amoebae and cysts were present in the culture of strain FUEN3 of *M. robusta* n. sp. The amoebae were the largest among *Monopylocystis* species (Fig. 1q–s). The cells appeared more vacuolated than the amoebae of the other strains; the vacuolization was also apparent in protargol-stained preparations (Fig. 2m, n). The nucleus of locomotive amoebae was located anteriorly in the granuloplasm during locomotion. Some cells possessed a villous-bulbous uroid with multiple short filaments (Fig. 1r, s). Cysts displayed typical morphology of *Monopylocystis* and were bigger than the cysts of the other species (Fig. 6h).

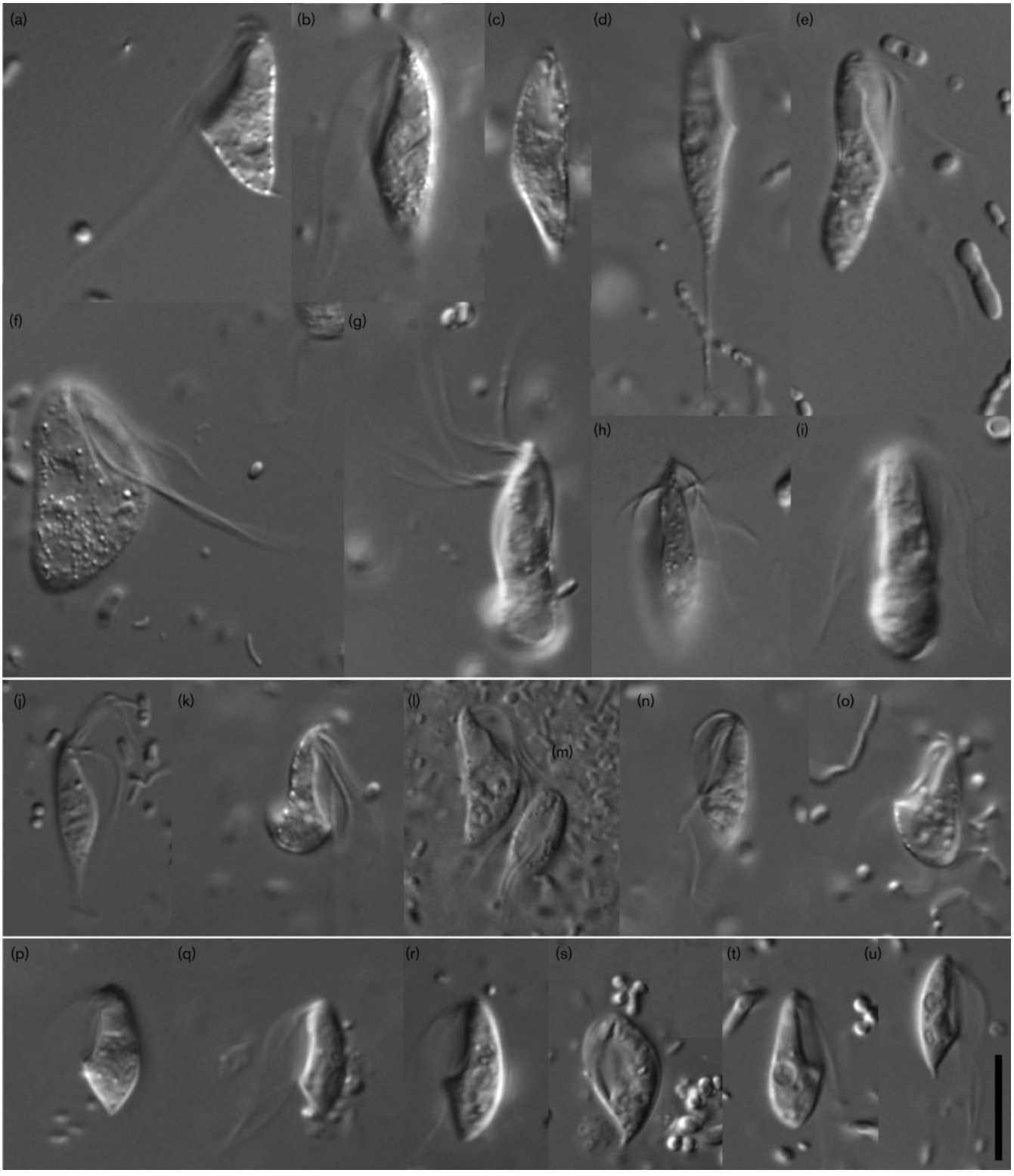

Fig. 4. Living flagellates of *Pseudoharpagon longus* n. sp. strain EVROS1I (a–i), *Pseudoharpagon tertius* n. sp. strain LAGOS1P (j–o), and *H. salinus* n. sp. strain TSUKIM (p–u). DIC. Bar, 10 μm.

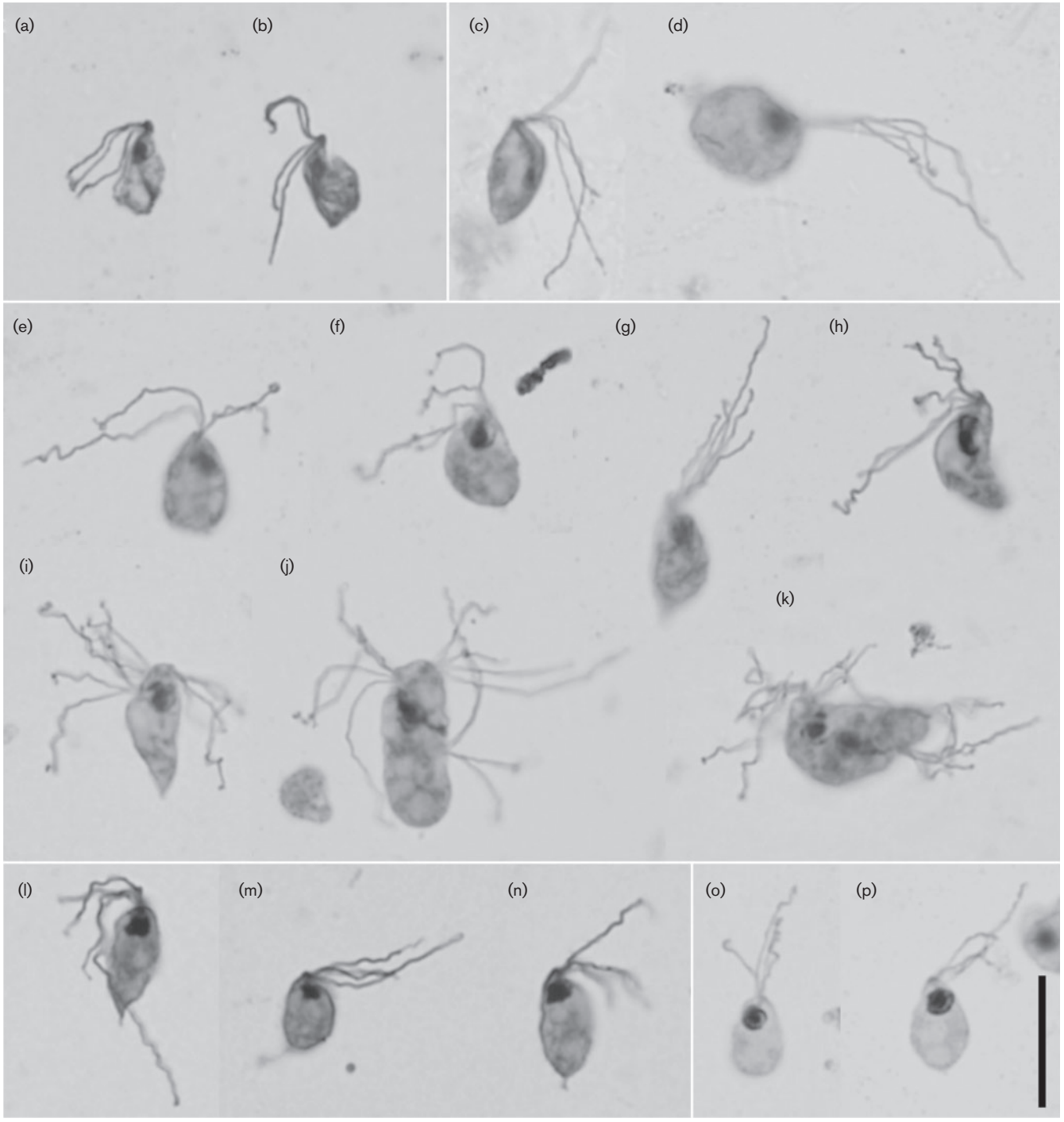

Fig. 5. Protargol-stained flagellates of *M. minor* n. sp. strain AND (a, b), *M. elegans* n. sp. strain EVROS1M (c, d), *Pseudoharpagon longus* n. sp. strain EVROS1I (e–k), *Pseudoharpagon tertius* n. sp. strain LAGOS1P (l–n) and *H. salinus* n. sp. strain TSUKIM (o, p). Bright field. Bar, 10 μm.

***Pseudoharpagon longus* n. sp.** Amoebae, flagellates and cysts were observed in the culture of strain EVROS1I. The amoebae were larger than the amoebae of most species of the genus *Monopylocystis* (Figs 1t–v, 2o, p, Table 2). The cytoplasm contained multiple tiny granules; the granules were abundant in both hyaloplasm and granuloplasm making the boundary between these two zones less apparent (Fig. 1t–v). The cells occasionally formed a bulbous or bulbous-villous uroid (Fig. 1v). The nuclear morphology was similar to that of species of the genus *Monopylocystis* (Figs 1t–v, 2o, p).

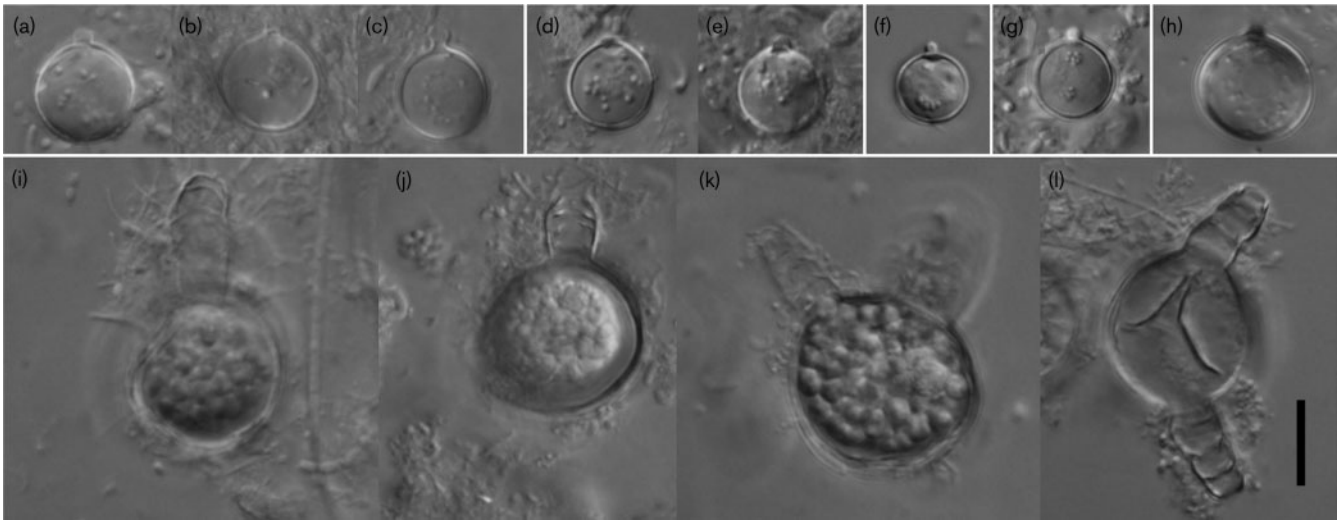

Fig. 6. Cysts of *M. visvesvarai* strains IGO3 (a), KORISSION3 (b) and TSUKIMV (c), *M. minor* n. sp. strains AND (d) and TINI (e), *M. disparata* n. sp. strain FUEN4 (f), *M. elegans* n. sp. strain EVROS1M (g), *M. robusta* strain FUEN3 (h) and *Pseudoharpagon longus* n. sp. strain EVROS1I (i–l). DIC. Bar, 10 μm.

The nucleus did not occupy a stable position in the granuloplasm in locomotive amoebae, similarly to *M. elegans* n. sp., and it was occasionally observed even in the uroid (Fig. 1v); it was not deformed during the cell locomotion.

The flagellates of *Pseudoharpagon longus* n. sp. were larger than that of the other known *Pseudoharpagon* species (Fig. 4a–i, Table 2). The cells were usually elongated, and a large variability in the shape of the posterior end was observed (compare Fig. 4a, d, e). *Pseudoharpagon longus* n. sp. differed from the other species of the family Psalteriomonadidae in the number of flagella. Cells with multiple flagella (Fig. 4f–i) occurred in the culture besides the tetrakont ones. The unusual flagellar number of *Pseudoharpagon longus* n. sp. was apparent in protargol-stained cells (Fig. 5e–k). From 30 randomly selected protargol-stained cells, only 23 % were tetrakont, whereas 57 % possessed five flagella and 20 % possessed more than five flagella. The flagella of the multiflagellate cells seemed to be arranged in at least two separate clusters consisting of approximately five flagella (Figs 4g–i, 5i–k). Most such cells possessed a single nucleus (Fig. 5i, j); occasionally, binucleate, multiflagellate cells were observed (Fig. 5k). The nucleus of living cells contained a thin peripheral layer of an uneven thickness (Fig. 4a–c, e). The structure of the nucleus and adjacent area appeared more complex in protargol-stained flagellates, though a peripheral dense layer was visible (Fig. 5f, h–k). The flagellates possessed a ventral groove that reached approximately one half of the cell (less in longer cells; Fig. 4d); the multiflagellate cells possessed two grooves, each being associated with a cluster of flagella (Fig. 4h, i).

The cysts occurring in the culture of strain EVROS1I were considerably larger than cysts of species of the genus *Monopylocystis*, and their morphology was unusual (Fig. 6i–l). They were rounded and usually produced two (one to four) long projections showing annulation or segmentation. The arrangement of the projections differed among individual cysts. Some projections had an aperture at the distal end (Fig. 6j, k), whereas the others seemed to be terminated by a cyst wall (Fig. 6i, l). The cysts contained a dense mass of relatively large globules.

***Pseudoharpagon tertius* n. sp.** Amoebae and flagellates were present in the culture of strain LAGOS1P; strain MURANO3 was lost before its morphology could be examined. The amoebae of LAGOS1P were quite rare in the culture, and their morphology is documented only in protargol-stained preparations (Fig. 2q, r). They displayed numerous thin pseudopodia, and the nuclear structure was similar to that of *Pseudoharpagon longus* n. sp. and species of the genus *Monopylocystis*. The flagellates were always tetrakont (Fig. 5l–n) and possessed a ventral groove reaching one half to two thirds of the cell, rarely almost its posterior end (Fig. 4j–o). They displayed certain variability in the shape of the posterior end of the cell, similarly to the other *Pseudoharpagon* species (compare Fig. 4j, k, l). The nucleus of living flagellates was similar to that of *Pseudoharpagon longus* n. sp. (Fig. 4l, n); protargol-stained nuclei were uniformly dark (Fig. 5l–n).

***Harpagon salinus* n. sp.** Flagellates, amoebae and cysts were observed in the culture of the strain TSUKIM. However, the amoebae and cysts were ascribed to *M. visvesvarai*, as they were morphologically consistent with the other isolates of this species (Figs 1g, 6c; see above), and SSU rDNA of this species was recovered from the culture together with SSU rDNA of *H. salinus* n. sp. By contrast, the flagellates were dissimilar to those of species of the genus *Monopylocystis* and

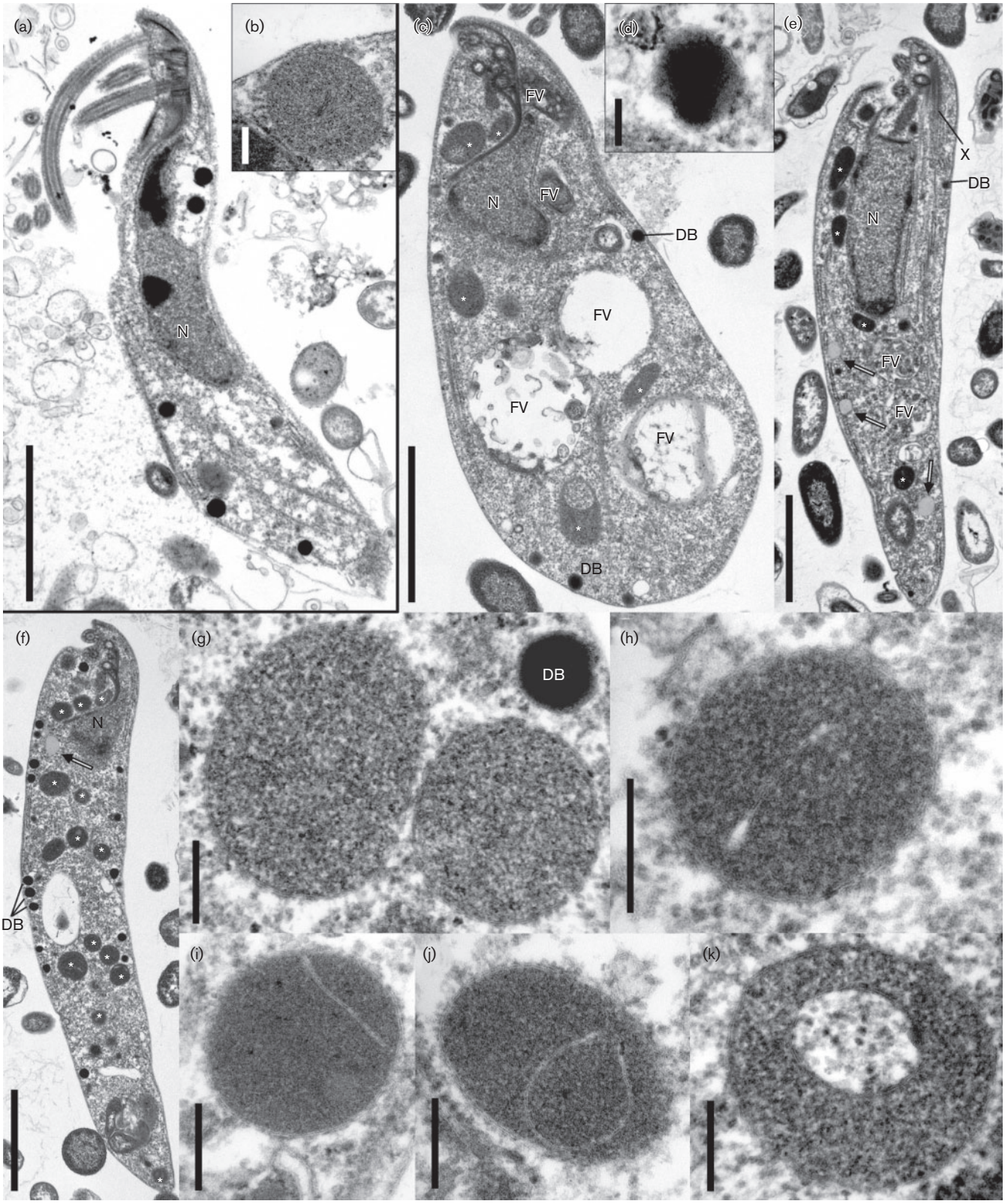

Fig. 7. Transmission electron micrographs of *Pseudoharpagon tertius* LAGOS1P (a, b) and *Pseudoharpagon pertyi* EVROS2N (c–k). Cell of *Pseudoharpagon tertius* in longitudinal section, lateral view (a); mitochondrion-related organelle of *Pseudoharpagon tertius* (b); cells of *Pseudoharpagon pertyi* in longitudinal sections, ventral view (c, f); detail of the dense body of *Pseudoharpagon pertyi* bounded by single membrane (d); possibly dividing cell of *Pseudoharpagon pertyi* in

longitudinal section, ventral view (e); mitochondrion-related organelles of *Pseudoharpagon pertyi*, section without visible cristae (g); mitochondrion-related organelle of *Pseudoharpagon pertyi* with a putative discoidal crista (h–j); cup-shaped mitochondrion-related organelle of *Pseudoharpagon pertyi* (k). Bars, 2 μm (a), 200 nm (b), 2 μm (c), 100 nm (d), 2 μm (e, f), 200 nm (g–k). DB, small dense body; FV, food vacuole; N, nucleus; X, unidentified microtubular structure; arrow, lipid droplets; asterisk (*), mitochondrion-related organelle.

resembled those of species of the genera *Harpagon* or *Pseudoharpagon* by the ventral groove, which reached only slightly past one half of the cell (Fig. 4p–u). The cells were smaller than cells of *Harpagon descissus* and *Harpagon schusteri*, were spindle-shaped, and their posterior end was usually more or less pointed. The nucleus of both living and protargol-stained cells was similar to that of species of the genera *Monopylocystis* and *Pseudoharpagon* (Figs 4p, 5o, p).

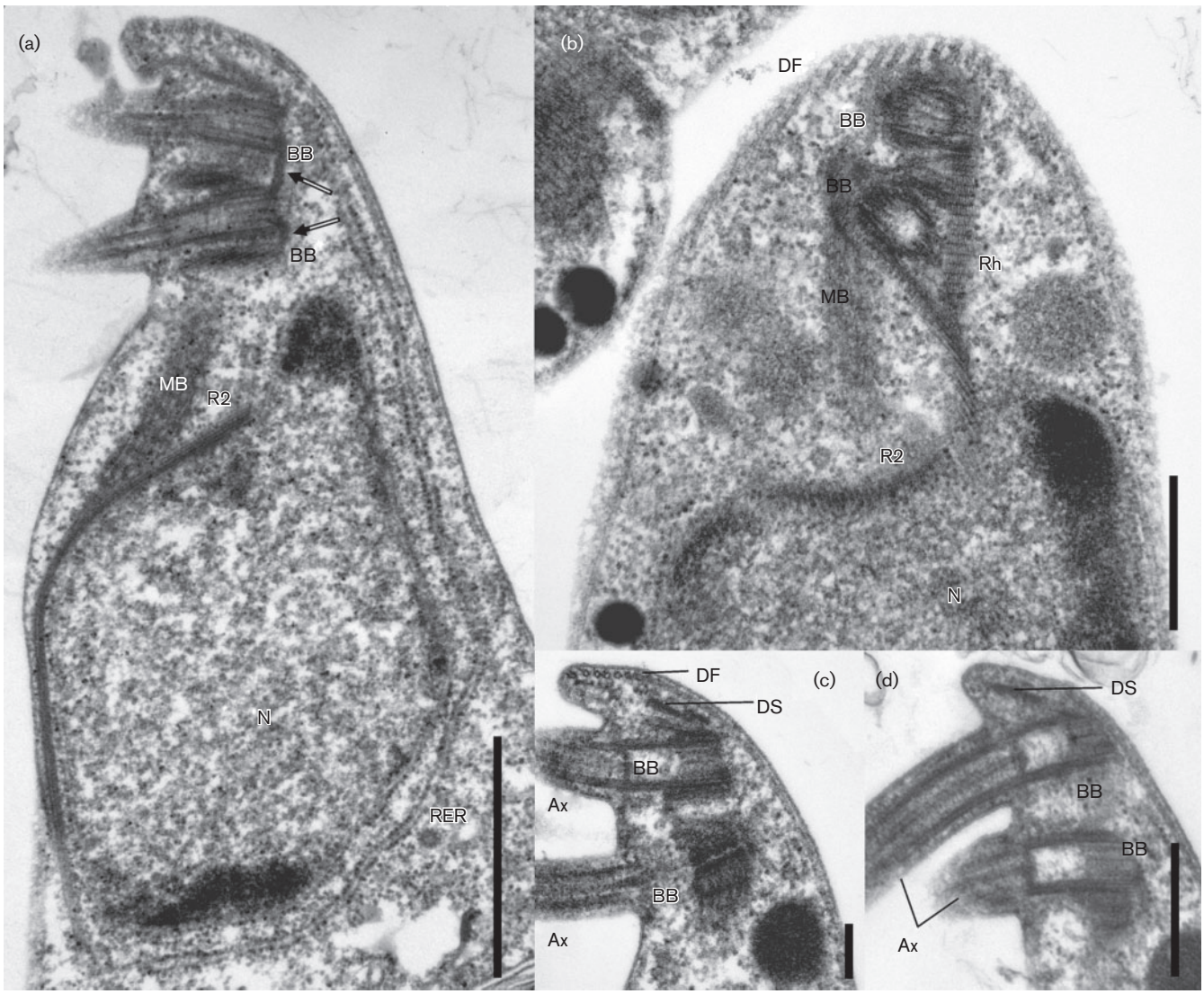

Fig. 8. Transmission electron micrographs of the flagellar apparatus of *Pseudoharpagon pertyi* EVROS2N. Anterior half of the cell in longitudinal section, lateral view, showing microfibrillar connection between posterior basal bodies and the nucleus (a); anterior part of the cell in an oblique section showing striated rhizoplast and basal bodies 1 and 2 (b); anterior part of the cell in longitudinal section from the lateral view (c, d). Bars, 1 μm (a), 500 nm (b), 200 nm (c), 500 nm (d). Ax, axoneme of the flagellum; BB, basal body of the flagellum; DF, dorsal fan of microtubules; DS, dense sheet; MB, microfibrillar bundle; N, nucleus; RER, rough endoplasmic reticulum; Rh, rhizoplast; R2, microtubular root 2; arrow, fibrous sheet below the proximal end of basal bodies.

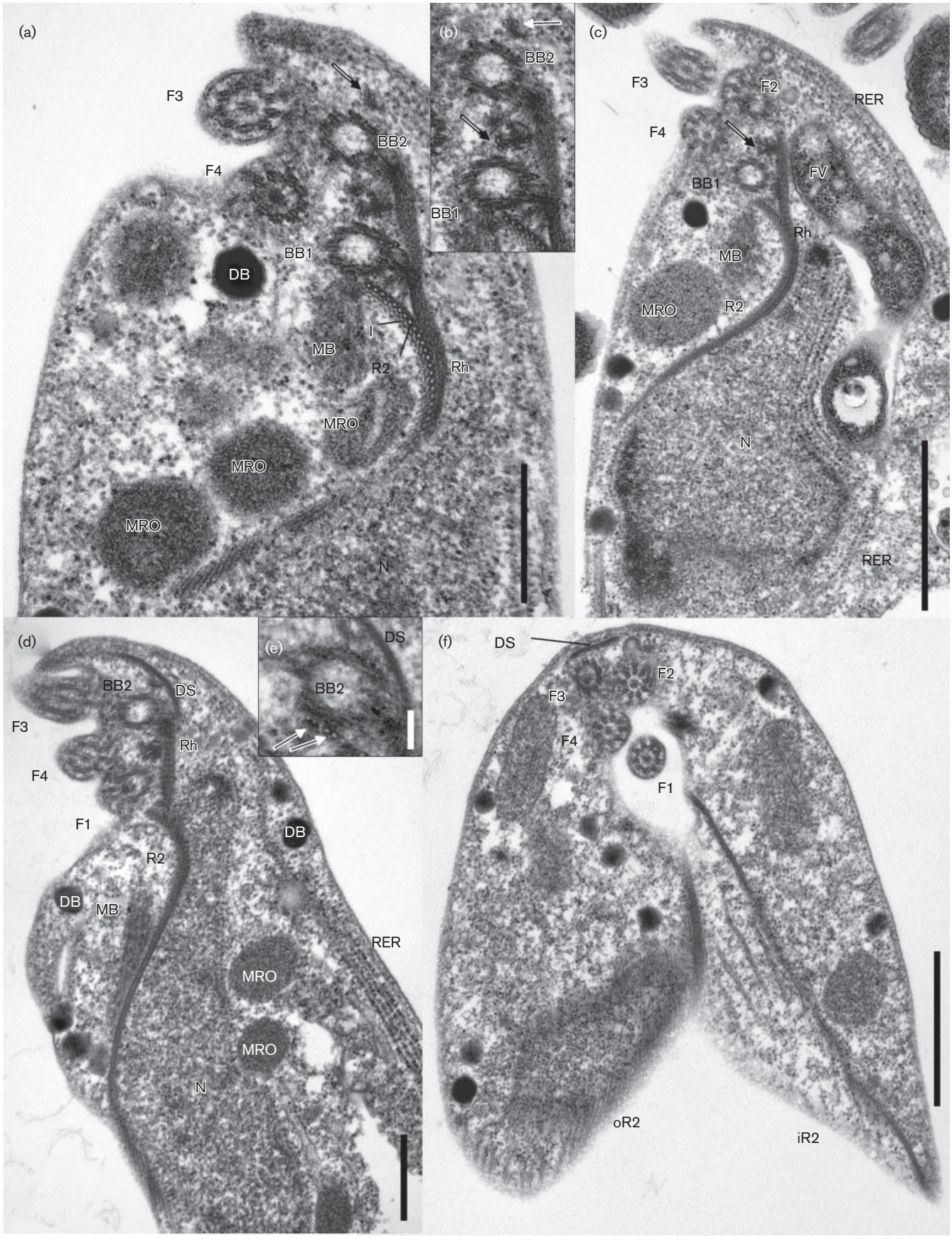
(a)
F3
BB2
F4
BB1
DB
I
MB
R2
Rh
MRO
MRO
MRO
N
(b)
BB2
BB1
(c)
F3
F2
RER
F4
FV
BB1
Rh
MB
MRO
R2
N
RER
(d)
BB2
DS
F3
Rh
F4
F1
DB
R2
DB
MB
RER
MRO
MRO
N
(e)
DS
BB2
(f)
DS
F2
F3
F4
F1
oR2
iR2

Fig. 9. Transmission electron micrographs of the flagellar apparatus of *Pseudoharpagon pertyi* EVROS2N. Anterior part of the cell in longitudinal section, ventral view, showing the flagellar apparatus (a) and detail of the basal bodies 1 and 2 with associated microtubules in another section of the same cell (b); anterior part of the cell in longitudinal section, ventral view, showing the flagellar apparatus (c); anterior part of the cell in longitudinal section, ventral view, showing the flagellar apparatus and connection between dense sheet and rhizoplast (d); basal body 2 with associated microtubules – the same cell as in previous image, but another section (e); oblique section through the anterior part of the cell where flagella arise (f). Bars, 500 nm (a, b), 1 μm (c), 500 nm (d), 100 nm (e), 1 μm (f). BB1, basal body of the oldest flagellum; BB2, basal body of the second oldest flagellum; DB, small dense body; DS, dense sheet; FV, food vacuole; F1–F4, flagella; iR2, inner part of microtubular root 2; MB, microfibrillar bundle; MRO, mitochondrion-related organelle; N, nucleus; RER, rough endoplasmic reticulum; Rh, rhizoplast; R2, microtubular root 2; oR2, outer part of microtubular root 2; arrow, microtubular fibres anchoring basal bodies 1 and 2.

Transmission electron microscopy of members of the genus *Pseudoharpagon*

We examined the cell structure of *Pseudoharpagon tertius* n. sp. (strain LAGOS1P) and *Pseudoharpagon pertyi* (strain EVROS2N). General terminology of ultrastructural elements typical for Excavata was adopted from Yubuki *et al.* (2013), who revised the universal terminology of the excavate flagellar apparatus (see Moestrup, 2000; Simpson, 2003). Terms of elements specific for Heterolobosea (microfibrillar bundle, rhizoplast) and labelling of flagella in the double-bikont flagellar apparatus were adopted from Brugerolle & Simpson (2004).

The terminology of the microtubular root labelled here as the R2 merits a brief comment. In the older literature, the root was named 'curved microtubule-organizing ribbon' (MTOR; Broers *et al.*, 1993) or 'right root' (e.g. Simpson & Patterson, 2001; Park & Simpson, 2011). Simpson (2003) proposed that the right root of excavates corresponds to root 1 (R1) in Moestrup's universal system (Moestrup, 2000). Thus, the right root of heterolobosean flagellates was erroneously labelled as root 1 (R1) instead of root 2 (R2) in several studies (Simpson, 2003; Brugerolle & Simpson, 2004; Pánek *et al.*, 2012).

***Pseudoharpagon tertius* n. sp.** Only a few cells of strain LAGOS1P were examined. Preliminary transmission electron microscopy showed that the nucleus was irregular, located apically, and with a thin projection at the anterior part (Fig. 7a). It contained at least two distinct aggregates of dense material (Fig. 7a) and was associated with rough endoplasmic reticulum (data not shown). Mitochondrion-related organelles (MROs) were rounded, 400–800 nm in diameter (Fig. 7b). Some MROs contained internal paracrystalline structures (Fig. 7b), but the quality of the sections was not sufficient to address the presence or absence of internal membranous structures observed in *Pseudoharpagon pertyi* (see below).

***Pseudoharpagon pertyi* strain EVROS2N.** The nucleus was pear-shaped, located anteriorly, below the basal bodies, and contained a peripheral layer of electron-dense material of uneven thickness. In both longitudinal- and cross-sections of the nucleus, two to four massive aggregations of dense material (parietal nucleoli) were observed (compare Figs 7c, e, 8a, 10c, e). The cells did not possess focused Golgi apparatus represented by stacked membrane-bounded sacs. MROs were in many copies per cell (Fig. 7f); they were rounded (Fig. 7g), sometimes cup-shaped (Fig. 7k), 400–700 nm in diameter, and bounded by a double membrane. Many of them contained a single membranous structure that closely resembled a discoidal crista (Fig. 7h–j). Rough endoplasmic reticulum surrounded the nucleus, but it was not associated with MROs. The cells usually contained food vacuoles with ingested bacteria (Fig. 7c, e). Small dense bodies bounded by a single membrane, 100–250 nm in diameter (Fig. 7d, g), were present under the cell membrane (Fig. 7f). Some cells contained lipid droplets (Fig. 7 e, f).

The flagellar apparatus of *Pseudoharpagon pertyi* was located subapically and was closely associated with the nucleus (Fig. 7c). Four basal bodies were arranged in two pairs. The pairs of basal bodies laid in tandem, basal bodies 1 and 4 formed a posterior pair, 2 and 3 formed the anterior one (Fig. 9a, c). The basal bodies were arranged nearly parallel to each other in a pair (Fig. 8a, c, d) with the proximal end lying on a 'pad' or fibrous sheet (Fig. 8a); they were 400–450 nm long. In a ventral view, the basal bodies laid rightwards. Axonemes of flagella displayed the typical '9 + 2' arrangement of microtubules and bore no vanes or paraxonemal structures (Figs 9f, 10f).

Microtubular root R2 anchored in the vicinity of basal body 1 (Fig. 9a). It was a curved row of approximately 55 microtubules that split into two unequal parts soon after the origin. The smaller left portion, iR2, consisted of approximately 15 microtubules; the right portion, oR2, consisted of approximately 40 microtubules. oR2 and iR2 supported the edges of the ventral groove (Fig. 10a, c, e, g). The concave side of R2 was supported by 'I fibre', a non-microtubular structure that appeared latticed in transverse sections (Fig. 9a). oR2 was associated with a non-microtubular string (Fig. 10b). The string may be homologous to the 'microfibrillar string S' reported from *Psalteriomonas lanterna* and *H. descissus*, where it acts as a nucleating centre for spaced microtubules reinforcing the floor of the ventral groove (Broers *et al.*, 1990; Brugerolle & Simpson, 2004).

The concave side of R2 was connected with the posterior pair of basal bodies via a thick microfibrillar bundle

(compare Figs 8a, b, 9a, c, d). A large striated fibre, the rhizoplast, connected the convex side of R2 and basal bodies 1 and 2. The rhizoplast displayed cross striation with periodicity of approximately 35 nm (Figs 8b, 9c, d) and longitudinal striation with periodicity of approximately 10 nm (Fig. 8b). The rhizoplast was associated with a sheet of dense material (Figs 8c, d, 9d), which ran from the dorsal side of the rhizoplast above basal bodies to the anterior tip of the cell (Fig. 9d).

Another microtubular root, composed of two microtubules, originated anteriorly from basal body 1 (Fig. 9c). Two or three microtubular fibres anchored in the vicinity of basal body 2. One of them, composed of two microtubules, was situated anteriorly (Fig. 9a, b); the other one or two, together composed of four or five microtubules, were located posteriorly in the same position as the fibre R2′ in *H. descissus* (Fig. 9e). No microtubular fibre associated with basal bodies 3 or 4 was observed; however, this part of the flagellar apparatus was not examined in detail.

The dorsal and lateral surfaces of the cell were supported by a fan of microtubules (dorsal fan; Figs 8c, 10f, h). The dorsal fan originated apically, above the basal bodies (Fig. 9f). A few microtubules, putatively derived from the dorsal fan, participated in the support of the right side of the feeding groove (Fig. 10c, d). Two groups of microtubules, collectively referred to as 'X', were observed in a single cell (Fig. 7e). They originated at basal bodies 1 and 2 and ran posteriorly.

Phylogenetic analysis of SSU rDNA

The SSU rDNA sequence of strain AND of *M. minor* n. sp. (but not strain TINI of the same species) and strain TSUKIM of *H. salinus* n. sp. included a single group I intron. It was 431 bp in length and inserted after position 1207 in AND (GenBank accession no. KF840521), and 853 bp in length after position 1245 in TSUKIM (KF840535). These positions were homologous to those of group I introns of the organisms '*Pseudomastigamoeba longifilum*' (AF011465), *Pleurostomum flabellatum* (DQ979962), *Acrasis helenhemmesae* (GU437219; its second intron) and *Pharyngomonas kirbyi* (HQ898857; its first intron). Tetramitia-specific helix 17_1 in the secondary structure of the SSU rDNA molecule was found in all newly determined sequences (not shown) except for strain COORONG of *M. disparata* n. sp., whose determined part of the sequence started in the helix E23_14.

The phylogenetic tree of Heterolobosea as inferred from SSU rDNA sequences is shown in Fig. 11. The overall phylogeny of Heterolobosea was consistent with previous analyses (Park & Simpson, 2011; Brown *et al.*, 2012; Pánek *et al.*, 2012; Park *et al.*, 2012; Harding *et al.*, 2013). Monophyletic clade VI of Tetramitia as defined by Pánek *et al.* (2012) was recovered and was statistically highly supported – maximum-likelihood bootstrap support (BS) 91, Bayesian posterior probability (BPP) 1. The family Psalteriomonadidae as defined by Pánek *et al.* (2012) appeared robustly monophyletic as well (BS 100, BPP 1). The family Psalteriomonadidae was closely related to a clade formed by the genera *Vrihiamoeba*, *Oramoeba* and *Stachyamoeba*, and by an uncultured heteroloboseid, WIM43; the monophyly of the clade (BS 70, BPP 0.98) as well as its relationship with the family Psalteriomanadidae (BS 52, BPP <0.9) was only poorly supported.

The family Psalteriomonadidae split into three robust clades (monophyly of each clade was supported by BS $\geqslant 94$, BPP 1), whose interrelationships were not resolved. The first clade was formed by strains of the genus *Pseudoharpagon*. *Pseudoharpagon pertyi* was sister to *Pseudoharpagon longus* n. sp. Genetic distance (p distance) between particular *Pseudoharpagon* species ranged between 0.228 and 0.269. The intraspecific genetic distance within *Pseudoharpagon pertyi* and *Pseudoharpagon tertius* n. sp. was 0.194 and 0.062, respectively. The second clade of the family Psalteriomonadidae was formed by *Sawyeria*, *Psalteriomonas* and two environmental sequences. The genera *Sawyeria* and *Psalteriomonas* were robustly monophyletic (BS 100, BPP 1) and appeared closely related (BS 91, BPP 1). The third clade of the Psalteriomonadidae was formed by the genera *Harpagon* and *Monopylocystis*. The genus *Harpagon* was robustly monophyletic (BS 100, BPP 1) and split into three lineages represented by *H. descissus*, *H. schusteri* and *H. salinus* n. sp., respectively; the former two species were closely related with maximum support. The mean genetic distance between *H. salinus* n. sp. and *H. descissus*, and *H. salinus* n. sp. and *H. schusteri* was 0.218 and 0.215, respectively.

The genus *Monopylocystis* appeared monophyletic, although its monophyly was unsupported (BS$<$50, BPP$<$0.9). The genus *Monopylocystis* split into six lineages represented by *M. visvesvarai*, *M. minor* n. sp., *M. disparata* n. sp., *M. similis* n. sp., *M. robusta* n. sp. and '*Pseudomastigamoeba longifilum*'. The relationships between the particular lineages were largely unresolved, except for the highly supported close relationship between *M. robusta* n. sp. and '*Pseudomastigamoeba longifilum*' (BS 100, BPP 1). The genetic distance between particular *Monopylocystis* species (when introns were removed) ranged between 0.056 (strain LAGOS1M of *M. disparata* n. sp. and strain EVROS1M of *M. similis* n. sp.) and 0.192 (strain TINI of *M. minor* n. sp. and '*Pseudomastigamoeba longifilum*'). Maximum intraspecific genetic distance was within *M. visvesvarai* (0.026). The genetic distance between *M. robusta* n. sp. and '*Pseudomastigamoeba longifilum*' was 0.120.

To examine relationships within the family Psalteriomonadidae more thoroughly, a separate analysis based on the second dataset was carried out (Fig. 12). The genus *Pseudoharpagon* was robustly monophyletic (BS 100, BPP 1), and the close relationship between *Pseudoharpagon pertyi* and *Pseudoharpagon longus* n. sp. was highly supported (BS 95, BPP 1). The clade of *Monopylocystis* and *Harpagon* received relatively high support (BS 86, BPP 1). Monophyly of the genus *Harpagon* and the close relationship between *H. descissus* and *H. schusteri* were

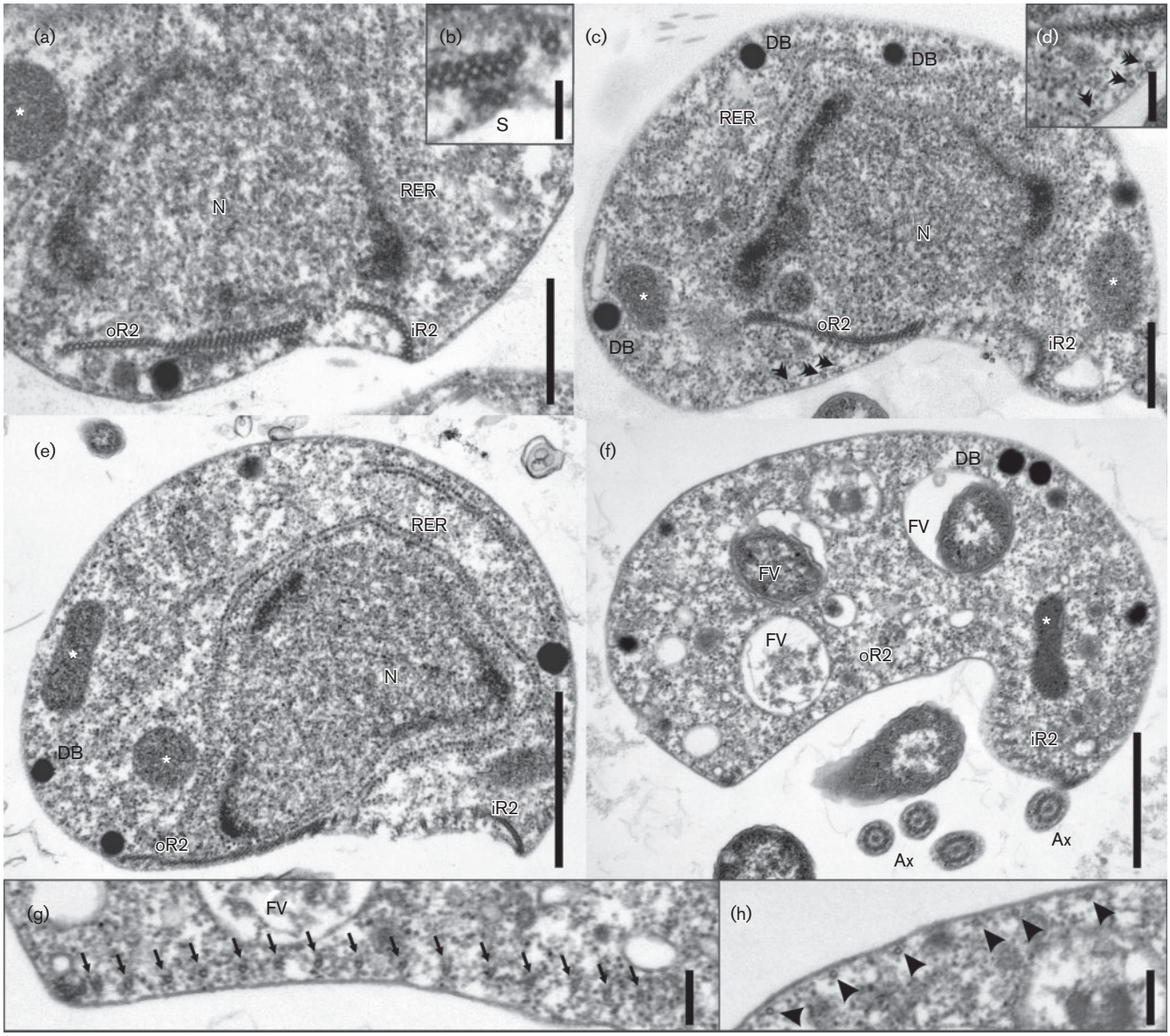

Fig. 10. Transmission electron micrographs of *Pseudoharpagon pertyi* EVROS2N. Anterior part of the cell in transverse section showing R2 after separation of oR2 and iR2 (a) and detail of putative microfibrillar string S (b); section posterior to (b) showing microtubules of dorsal fan supporting the feeding groove (c); detail of these microtubules (d); section posterior to (c) showing nucleus surrounded by rough endoplasmic reticulum (e); transverse section through the cell below the nucleus showing widened feeding groove and dorsal fan of microtubules (f); detail of some oR2 microtubules (rotated image when compared with f) (g); detail of some microtubules of the dorsal fan (h). Bars, 500 nm (a), 100 nm (b), 500 nm (c), 200 nm (d), 1 μm (e, f), 200 nm (g, h). Ax, axonemes of flagella; S, putative microfibrillar string S; DB, small dense body; FV, food vacuole; iR2, inner part of microtubular root 2; N, nucleus; RER, rough endoplasmic reticulum; oR2, outer part of microtubular root 2; arrows, microtubules of R2; arrowheads, microtubules belonging to dorsal fan; asterisks, mitochondrion-related organelles; double arrowheads, microtubules of dorsal fan supporting the feeding groove.

recovered with maximum support. Monophyly of the genus *Monopylocystis* remained only weakly supported (BS 62, BPP 0.99). The relationships between particular *Monopylocystis* species were better resolved than in the analysis based on the first dataset. *M. robusta* n. sp. and '*Pseudomastigamoeba longifilum*' formed a robust clade that was sister to the remaining *Monopylocystis* species. *M. disparata* n. sp. and *M. similis* n. sp. formed a moderately supported clade (BS 79, BPP 0.99). *M. minor* n. sp. was closely related to *M. visvesvarai* (BS 82, BPP 0.99).

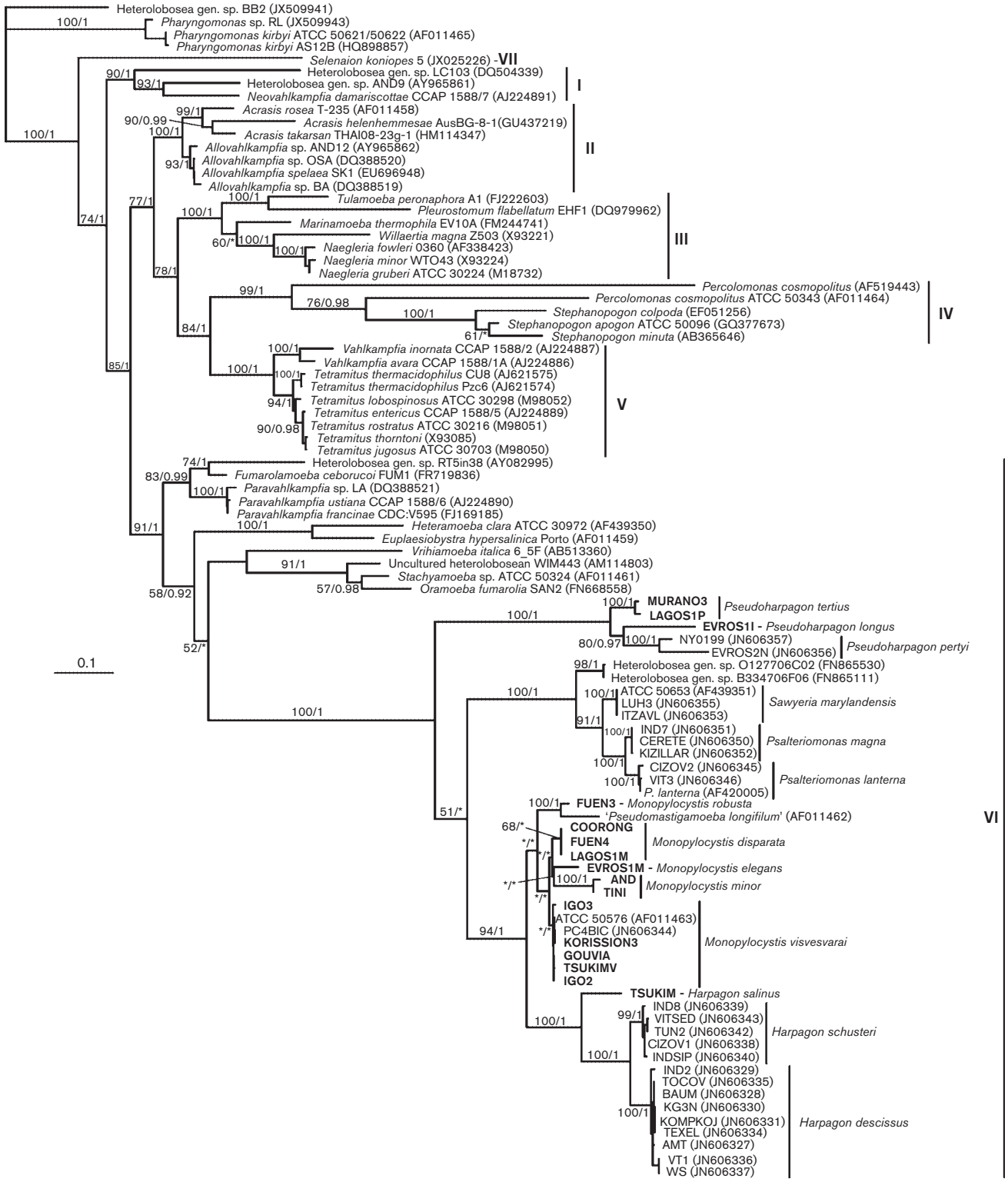

Fig. 11. Unrooted phylogenetic tree of Heterolobosea based on SSU rDNA sequences. The tree was reconstructed by the maximum-likelihood method in RAxML (GTRGAMMAI model). Values at branches represent statistical support as bootstrap values (RAxML)/posterior probabilities (MrBayes). Support values below 50/0.90 are represented by an asterisk (*). Seven main clades of the subphylum Tetramitia are labelled. Newly determined sequences are in bold type. Bar, 0.1 substitutions per nucleotide position.

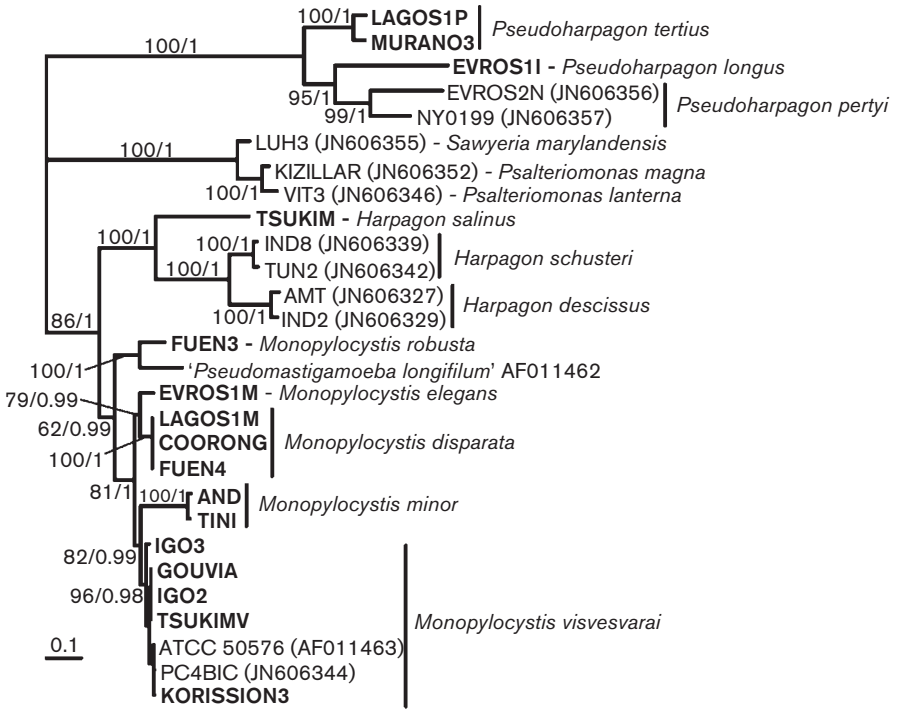

Fig. 12. Unrooted phylogenetic tree of the family Psalteriomonadidae based on SSU rDNA sequences. The tree was reconstructed by the maximum-likelihood method in RAxML (GTRGAMMAI model). Values at branches represent statistical support as bootstrap values (RAxML)/posterior probabilities (MrBayes). Newly determined sequences are in bold type. Bar, 0.1 substitutions per nucleotide position.

Discussion

Species identities of strains

Recent discoveries of multiple heterolobosеid species and genera suggest that the diversity of this group, especially in non-canonic environments, is only poorly known (e.g. De Jonckheere *et al.*, 2011a, b; Brown *et al.*, 2012; Pánek *et al.*, 2012; Park *et al.*, 2012; Harding *et al.*, 2013). Our data indicate that this is also true for anaerobic heterolobosеids. We have isolated and cultured 16 strains from brackish/marine/saline environments. To assess the phylogenetic position of the newly obtained isolates, we determined and analysed their SSU rDNA sequences, currently the only phylogenetic marker with a sufficient sampling of Heterolobosea. Phylogenetic analyses showed that all the strains belong to the family Psalteriomonadidae *sensu* Pánek *et al.* (2012). Based on light-microscopic morphology and phylogenetic position, our isolates belong to eight species.

We assign most (twelve) strains to the genus *Monopylocystis*. O'Kelly *et al.* (2003) defined the genus *Monopylocystis* by the nuclear structure of the amoeba and morphology of the cyst. Later, Pánek *et al.* (2012) discovered the flagellate stage of *Monopylocystis*. However, only two strains of *Monopylocystis* amoebae (from which only one formed cysts) and a single strain of flagellates were available until the present study. Our new data allow a more precise description of all three stages of members of the genus *Monopylocystis*. The amoeba is taxonomically the least important stage of heterolobosеids (see Pánek & Čepička, 2012). Nevertheless, the amoeba of *Monopylocystis* is distinct enough to be easily recognized. It differs from amoebae of species of the genus *Psalteriomonas* and *Sawyeria marylandensis* in the structure of the nucleus. The nucleolar material of *Monopylocystis* amoebae is peripheral, distributed in a thin layer beneath the nuclear membrane (Smirnov & Fenchel, 1996; O'Kelly *et al.*, 2003). By contrast, amoebae of *Sawyeria marylandensis*, *Psalteriomonas lanterna* and *Psalteriomonas magna* possess one or two parietal nucleoli in the nucleus (O'Kelly *et al.*, 2003; Pánek *et al.*, 2012). Although amoebae of *Pseudoharpagon longus* n. sp. possess the same nuclear structure as amoebae of species of the genus *Monopylocytis*, the latter species is unique by the absence of clear demarcation between hyaloplasm and granuloplasm. The flagellates of species of the genus *Monopylocystis* possess a single longitudinal feeding groove that occupies almost the whole ventral side. A similar (although somewhat shorter) ventral groove was described in two species of the genus *Psalteriomonas*, *Psalteriomonas lanterna* and *Psalteriomonas vulgaris* (Broers *et al.*, 1990, 1993). However, the genera *Monopylocystis* and *Psalteriomonas* are not closely related, and in phylogenetic trees of SSU rDNA they are separated by at least the genus

Harpagon, whose ventral groove is much shorter. Finally, we describe here cysts of all known *Monopylocystis* species except for *M. anaerobica*. All display the same morphology, i.e. a single prominent pore with a plug, and differ only slightly in diameter.

We classify our strains of the genus *Monopylocystis* into five species. With at least six species in total (we did not isolate *M. anaerobica*), *Monopylocystis* becomes the largest genus among the Psalteriomonadidae. Particular *Monopylocystis* species are morphologically similar to each other, but significant differences can be found. Five strains belong to the type species *M. visvesvarai*. Amoebae of *M. visvesvarai* strains are variable in length and width, suggesting that cell diameters alone are not good taxonomic markers. Unlike the type strain, many amoebae of all novel *M. visvesvarai* strains produce a uroid, sometimes with filaments of various lengths. Presence/absence of the uroid was important for O'Kelly *et al.* (2003) to distinguish between their newly described species *M. visvesvarai* and *M. anaerobica* (then known as *Vahlkampfia anaerobica*; Pánek *et al.*, 2012 transferred it to the genus *Monopylocystis*). The second important characteristic was the shape of the nucleus, which was reported to be more angular in the latter species (O'Kelly *et al.*, 2003). However, we show here that the nucleus of all *Monopylocystis* species except for *M. elegans* n. sp. is deformed to some extent during the locomotion. Moreover, the nuclear morphology of *M. visvesvarai* and *M. anaerobica* seems, in fact, to be quite similar (compare Figs 1 and 2 in O'Kelly *et al.*, 2003 with Fig. 1–3 in Smirnov & Fenchel, 1996). The presence/absence of the uroid and shape of the nucleus cannot, therefore, be considered important in this case. By contrast, *M. anaerobica* differs from *M. visvesvarai* and all other *Monopylocystis* species by its ability to form rayed floating cells. The rayed floating form thus remains the only feature that defines *M. anaerobica*. However, if the rayed form is discovered in *M. visvesvarai* in the future, the two names will be synonymized with *M. anaerobica* (Smirnov & Fenchel, 1996) having priority.

Strains of *M. minor* n. sp., AND and TINI, formed a clade that was closely related to *M. visvesvarai* in the analysis without non-psalteriomonadid outgroups. Their amoebae were very similar to the amoebae of *M. visvesvarai*, although they were slightly smaller, and the nucleus seemed to be more deformed during the rapid locomotion. Importantly, flagellates of both strains were considerably smaller than those of *M. visvesvarai* (approx. 11 vs 16 μm). The small flagellate together with the genetic distance of 0.117 from *M. visvesvarai* is, in our opinion, sufficient to classify strains AND and TINI into a separate species.

M. elegans n. sp. was represented by strain EVROS1M. Its amoeba differs strikingly from all other *Monopylocystis* species in having the nucleus located deeper in the granuloplasm in locomotive cells. The cells were observed repeatedly and always displayed this behaviour. The nucleus is also much less deformed during the locomotion and stays approximately spherical. Since no transmission electron microscopy data from *M. elegans* n. sp. are available, it is impossible to decide how the shape of the nucleus is preserved; it may be surrounded by a thicker layer of the endoplasmic reticulum than in the other species. The flagellates of *M. elegans* n. sp. are typically slightly larger than those of *M. visvesvarai*, much smaller than those of *M. disparata* n. sp., and are considerably more variable in size.

M. disparata n. sp., represented by strains LAGOS1M and FUEN4, was closely related to *M. elegans* n. sp., the genetic distance between the two species being 0.057. Unexpectedly, the two strains of *M. disparata* n. sp. were morphologically inconsistent with each other despite having almost identical SSU rDNA sequences (single nucleotide difference). The culture of FUEN4 contained amoebae and cysts that were the smallest among members of the genus *Monopylocystis*, whereas the only observed form of LAGOS1M was a flagellate, which, in turn, was the largest one. The culture LAGOS1M was derived from a sample that originally also contained *Pseudoharpagon tertius* n. sp. besides *M. disparata* n. sp. Later, we successfully separated the two species, and two cultures were created: LAGOS1M with *Monopylocystis*, and LAGOS1P with *Pseudoharpagon*. The culture LAGOS1M was accidentally lost after several tens of passages. Before that, its SSU rDNA had been sequenced twice independently from a purified PCR product; it was also cloned into a vector, and two clones were partially sequenced to confirm the species identity. Similarly, SSU rDNA of the culture of strain FUEN4 was sequenced three times independently during 2012. From our results, we infer that both LAGOS1M and FUEN4 contained only a single species of the family Psalteriomonadidae, *M. disparata* n. sp. FUEN4 and LAGOS1M are different strains, although with almost identical SSU rDNA sequences. Therefore, we cannot rule out the possibility that a potential undiscovered flagellate of FUEN4 would be small, while a potential amoeba of LAGOS1M would be large. In that case, the size of the cell would not be an appropriate taxonomic marker for *M. disparata* n. sp. Nevertheless, the amoeba of FUEN4 differs from the other *Monopylocystis* amoebae not only in size, but also in the form of the uroid, which is large and almost always bears very long, often multiple, filaments. In addition, it differs from the closely related *M. elegans* n. sp. in the position of the nucleus.

Strain FUEN3 clearly represents a separate species of the family Psalteriomonadidae on the basis of its phylogenetic position. Together with the organism represented by the sequence AF011462 and sometimes referred to as '*Pseudomastigamoeba longifilum*' (Nikolaev *et al.*, 2004), it appeared closely related to a clade formed by the remaining *Monopylocystis* species, but the relationship remained unsupported. We, in fact, cannot exclude the possibility that *M. robusta* n. sp. is closely related to the genus *Harpagon*, or that it represents a sister lineage to *Harpagon*+*Monopylocystis*. The amoebae and cysts of strain FUEN3 are morphologically fully consistent with the genus *Monopylocystis*. It is, in turn, impossible to compare the morphology of FUEN3 with that

of the genus *Harpagon*. Therefore, we conservatively place the organism into the genus *Monopylocystis* and describe it as *M. robusta* n. sp. In addition to the phylogenetic position, it can be differentiated from the other species of the genus by its considerably larger amoebae and cysts. The close relative of *M. robusta* n. sp., '*Pseudomastigamoeba longifilum*', very likely represents a separate *Monopylocystis* species due to its large genetic distance from *M. robusta* n. sp.

The genera *Harpagon* and *Pseudoharpagon* were described by Pánek *et al.* (2012). Members of both genera are known as pure flagellates; neither amoebae nor cysts have been reported previously. Although the two genera are not closely related in SSU rDNA trees, they are morphologically quite similar and not readily distinguishable from each other (Pánek *et al.*, 2012). The slight differences between members of the genera *Harpagon* and *Pseudoharpagon* lie in the extent of the ventral groove (up to one half of the cell length in the former vs one half to two thirds in the latter), cell length (approx. 14 vs 17 μm), variability of cell shape (cells of *Harpagon* are more pleomorphic than those of *Pseudoharpagon*), and habitat (freshwater vs marine/brackish anoxic sediments).

Strain TSUKIM was isolated from an inland salt marsh and is placed here into the genus *Harpagon* as a novel species, *H. salinus* n. sp. Although flagellates, amoebae and cysts appeared in the culture, the amoebae and cysts were ascribed to *M. visvesvarai* strain TSUKIMV (see above), and only the flagellates will be further considered. The morphology of strain TSUKIM was consistent with the genus *Pseudoharpagon* (longer ventral groove, relatively less variable cell shape, and smaller diameter of the cells). This taxonomic assignment was further supported by the habitat from which it was isolated and by the fact that it did not survive in the freshwater-based medium ATCC 802. Surprisingly, TSUKIM formed a robust clade with members of the genus *Harpagon* instead of those of the genus *Pseudoharpagon* in SSU rDNA trees. This indicates that the morphological distinction between these two genera proposed by Pánek *et al.* (2012) is no longer valid, and the genus *Harpagon* can be currently distinguished from the genus *Pseudoharpagon* only by phylogenetic position. Besides the aforementioned morphological and ecological distinctiveness of *H. salinus* n. sp. among members of the genus *Harpagon*, it differs from the other two described *Harpagon* species by the structure of the nucleus. Although the nuclei of neither *H. descissus* nor *H. schusteri* were studied in detail, they obviously contained several parietal nucleoli (Fig. 2f in Brugerolle & Simpson, 2004; Fig. 6F, 7D in Pánek *et al.*, 2012). By contrast, the nucleus of *H. salinus* n. sp. is morphologically similar to that of species of the genus *Monopylocystis* (and amoebae of species of the genus *Pseudoharpagon*, see below).

The phylogenetic position of the genus *Pseudoharpagon* remains uncertain. It forms a separate lineage of the family Psalteriomonadidae in SSU rDNA trees and does not seem to be specifically related to the morphologically similar genus *Harpagon*. Pánek *et al.* (2012) isolated two strains of heterolobosseid flagellates, which they accommodated in the newly described species *Pseudoharpagon pertyi* despite the large genetic distance between them. We follow Pánek *et al.* (2012) and consider the two strains conspecific, because they are closely related and seem to be morphologically identical. We isolated three additional strains that cluster with the genus *Pseudoharpagon* in SSU rDNA trees. Strains LAGOS1P and MURANO3 are placed into a novel species, *Pseudoharpagon tertius* n. sp. Although cells of LAGOS1P are morphologically hardly distinguishable from *Pseudoharpagon pertyi*, the two organisms are phylogenetically separated from each other by clearly distinct *Pseudoharpagon longus* n. sp. In addition to the flagellates, amoebae were observed in the culture of LAGOS1P. Although their species identity remains uncertain, they were morphologically similar to amoebae of *Pseudoharpagon longus* n. sp. (see below) and might thus belong to *Pseudoharpagon tertius* n. sp.

Strain EVROS1I displays an unusual morphology and clearly represents a separate species, here described as *Pseudoharpagon longus* n. sp. Amoebae, flagellates and cysts have been observed in the culture, the latter two being reported for the first time for the genus. The amoebae morphologically resemble members of the genus *Monopylocystis*, particularly because of the structure of the nucleus. On the other hand, the granulation of the anterior cytoplasm makes them easily recognizable. The flagellates of *Pseudoharpagon longus* n. sp. are considerably longer than those of the other *Pseudoharpagon* species. Their ventral groove often does not extend beyond the half of the cell, which further complicates morphological distinction between members of the genera *Harpagon* and *Pseudoharpagon*. The most prominent feature distinguishing *Pseudoharpagon longus* n. sp. from the other members of the family Psalteriomonadidae is the presence of five flagella in the majority of the flagellates; mastigonts of the other psalteriomonadids are invariably tetrakont (the sixteen flagella of *Psalteriomonas lanterna* are arranged into four separate mastigonts). The multiflagellate cells of *Pseudoharpagon longus* n. sp. probably represent dividing forms. If true, it means that the flagellar apparatus is restored prior to the nuclear division in this species. This is in contrast with a single known dividing cell of *Pseudoharpagon pertyi* (Fig. 9M in Pánek *et al.*, 2012), in which the nuclear division preceded duplication of flagella.

The cysts present in the culture of EVROS1I displayed a unique morphology, non-comparable to cysts of *Monopylocystis* or any other heterolobosseid. Although we have observed neither encystation nor excystation in the culture, we are convinced that the cysts truly belong to the life cycle of *Pseudoharpagon longus* n. sp. They have been constantly present in the culture of EVROS1I for more than a hundred passages. Moreover, the only contaminating eukaryotic organism in the culture was *Andalucia incarcerata*. It has quite small cells measuring about 7 μm (Bernard *et al.*, 2000). Although cysts of *A. incarcerata* are

unknown, we expect them to also be small, because cysts of the closely related species *Andalucia godoyi* are less than 5 μm in diameter (Fig. 4 in Lara *et al.*, 2006).

Ultrastructure of members of the genus *Pseudoharpagon*

Most heteroloboseid flagellates are tetrakonts with two pairs of flagella in a single mastigont; each pair bears a similar set of microtubular roots (Brugerolle & Simpson, 2004; Park & Simpson, 2011). Cells of *Pharyngomonas kirbyi*, a member of the subphyla Pharyngomonada, possess a plesiomorphic flagellar apparatus with R1/C fibre system, having duplicated R1/C and R2/I systems, and a possible homologue of the B fibre of other excavates (Park & Simpson, 2011). Members of the subphylum Tetramitia have lost the R1/C system. The role of R1, which normally supports the left side of the feeding groove, was assumed by an expanded R2 in the Tetramitia. The flagellar apparatus of the genus *Stephanopogon* has been substantially modified and does not resemble that of other heteroloboseids (Yubuki & Leander, 2008).

The structure of the flagellar apparatus of members of the family Psalteriomonadidae is well documented thanks to the comprehensive studies of *Psalteriomonas lanterna*, *Psalteriomonas vulgaris* and *H. descissus* (Broers *et al.*, 1990, 1993; Brugerolle & Simpson, 2004); limited ultrastructural data are also available from the flagellates of *M. visvesvarai* (Pánek *et al.*, 2012). These species display almost invariable structure of the flagellar apparatus, generally similar to that of other tetramitia. The cells of psalteriomonadids display a characteristic harp-shaped structure constituted by the microfibrillar bundle and R2 (Broers *et al.*, 1990, 1993; Cavalier-Smith, 1993; Pánek *et al.*, 2012). Although the structure was regarded as a unique feature of the Psalteriomonadidae (Pánek *et al.*, 2012), it is also present in almost identical form in *Percolomonas cosmopolitus* (Fenchel & Patterson, 1986).

Pseudoharpagon is the last genus of the family Psalteriomonadidae with known flagellate stage whose ultrastructure has not yet been convincingly reported. Bernard *et al.* (2000) published a single transmission electron micrograph of a heteroloboseid flagellate, which they identified as *Percolomonas descissus* (now *H. descissus*). Pánek *et al.* (2012) tentatively affiliated the organism with the genus *Pseudoharpagon* on the basis of its gross morphology and marine origin. However, its true identity is unclear since the diversity of flagellate psalteriomonadids in marine/saline environments is certainly greater than assumed by Pánek *et al.* (2012) – this is also shown by our discovery of *H. salinus* n. sp. We investigated the ultrastructure of the type strains of *Pseudoharpagon pertyi* and *Pseudoharpagon tertius* n. sp. Although the data obtained from the latter species were too limited to draw any conclusions, the results from *Pseudoharpagon pertyi* allowed comparison of its ultrastructure with the other members of the Psalteriomonadidae. The flagellar apparatus of *Pseudoharpagon pertyi* is very similar to that of other psalteriomonadids in every essential detail, including the presence of the harp-shaped structure. By contrast, *Pseudoharpagon pertyi* displays several uncommon features. Its basal bodies are underlain by a fibrous sheet. A similar sheet was documented in the aforementioned psalteriomonadid ('electron-dense pad'; Fig. 4F in Bernard *et al.*, 2000) and is likely to be present also in *H. descissus* (Fig. 1l, o in Brugerolle & Simpson, 2004). Interestingly, this structure was proposed as a synapomorphy of the unrelated heteroloboseid lineage Percolatea, which comprises the genera *Percolomonas* and *Stephanopogon* (Yubuki & Leander, 2008). The simultaneous presence of the sheet and harp-like structure in the family Psalteriomonadidae and class Percolatea could mean that the structures originated early in the evolution of the subphylum Tetramitia, or that the two lineages are specifically related.

In a single cell of *Pseudoharpagon pertyi*, we observed two conspicuous groups of microtubules, collectively referred to as the structure 'X'. Microtubules in a similar position were described from *Psalteriomonas lanterna* (Broers *et al.*, 1990), but the homology of the structures is questionable. The function of 'X' in *Pseudoharpagon pertyi* is unknown, but is possibly connected to initial steps of cell division.

All members of the family Psalteriomonadidae are anaerobic and possess acristate MROs that have properties of hydrogenosomes (O'Kelly *et al.*, 2003; Brugerolle & Simpson, 2004; de Graaf *et al.*, 2009; Barberà *et al.*, 2010; Pánek *et al.*, 2012). MROs of the Psalteriomonadidae display considerable variability in size and shape, both within and between species. They are globular (*M. visvesvarai*), cup-shaped (*Sawyeria marylandensis*), or irregular (*Psalteriomonas* spp.). The MROs of three known *Psalteriomonas* species form an aggregate with symbiotic prokaryotes, presumably methanogenic archaea (Broers *et al.*, 1990, 1993; Pánek *et al.*, 2012). Besides the aggregated MROs, *Psalteriomonas lanterna* and *Psalteriomonas magna* also possess individual MROs in the same cell (de Graaf *et al.*, 2009; Pánek *et al.*, 2012). Most MROs of *Pseudoharpagon pertyi* were globular, although a few cup-shaped MROs, resembling those of *Sawyeria marylandensis*, were observed. Interestingly, the MROs of *Pseudoharpagon pertyi* often contained a single structure that closely resembled a discoidal crista. Discoidal cristae are relatively rare among the eukaryotes and, notably, are a typical feature of heteroloboseids (Page & Blanton, 1985). If the structure is homologous to the mitochondrial cristae of the aerobic Heterolobosea, it might indicate a more complex mitochondrial metabolism of *Pseudoharpagon pertyi* in comparison with other members of the Psalteriomonadidae. However, the possibility of artefacts formed during fixation has to be taken into consideration, and the presence of the crista-like structure within MROs of *Pseudoharpagon pertyi* has yet to be confirmed.

In addition to MROs, smaller, electron-dense bodies bounded by a single membrane were relatively abundant in the peripheral cytoplasm of members of the genus

Pseudoharpagon. Such organelles have not been described in the other members of the Psalteriomonadidae, but similar structures were found in cells of unrelated *Pharyngomonas kirbyi* and *Selenaion koniopes* (Park & Simpson, 2011; Park *et al.*, 2012). In the former species, the bodies were putatively classified as peroxisomes. The dense bodies of *Selenaion koniopes* ('B bodies' in Park *et al.*, 2012) were assumed to be distinct from the peroxisomes, and their function is unclear.

Taxonomic summary

Eukaryota: Excavata: Discoba: Heterolobosea: Tetramitia: Psalteriomonadidae Cavalier-Smith, 1993.

Genus *Monopylocystis* O'Kelly, Silberman, Amaral Zettler, Nerad & Sogin 2003

Diagnosis: Marine, brackish or living in inland salt marshes. Uninucleate. Flagellate, cyst and amoeba stages ancestrally present, one or two stages unknown in some species. Amoeba with distinctive demarcation between granuloplasm and hyaloplasm. Flagellate tetrakont, with a ventral groove reaching the posterior end of the cell. Nucleolar material peripheral, distributed in a thin layer beneath the nuclear membrane. Cyst possessing single pore plugged with gelatinous material.

Type species: *Monopylocystis visvesvarai* O'Kelly, Silberman, Amaral Zettler, Nerad & Sogin 2003 by monotypy.

Included species: *M. anaerobica* (Smirnov & Fenchel, 1996)=*Vahlkampfia anaerobica* Smirnov & Fenchel 1996; *M. visvesvarai* O'Kelly, Silberman, Amaral Zettler, Nerad & Sogin 2003; *M. disparata* n. sp.; *M. elegans* n. sp.; *M. minor* n. sp.; *M. robusta* n. sp.

Monopylocystis minor n. sp.

urn:lsid:zoobank.org:act:12AFACB8-76B9-4859-BC60-4F3FEB47AA76.

Diagnosis: *Monopylocystis* with locomotive amoeba 10 to 31 (mean 17.4) μm long. Nucleus located in the anterior part of the granuloplasm and deformed during locomotion. Floating form globular. Flagellate smaller than in other *Monopylocystis* species, 10 to 14 (mean 10.7) μm long. Cyst with diameter 7 to 12 (mean 8.7) μm.

Type locality: Elephant beach, Havelock Island, Andaman Islands, India. 12° 00′ N 92° 56′ E.

Habitat: Anoxic marine coastal sediments.

Syntype: Protargol preparations of the culture AND with *M. minor* and an unidentified flagellate, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 9/30–9/32.

Etymology: *minor* (Latin adj.) smaller. Named after the small size of the flagellate.

Monopylocystis disparata n. sp.

urn:lsid:zoobank.org:act:9A048CD5-FA9F-48AA-83EE-05427AD5E725.

Diagnosis: *Monopylocystis* with locomotive amoeba smaller than in other species, 9 to 31 (mean 16.4) μm long. Nucleus located in the anterior part of the granuloplasm and deformed during locomotion. Uroid with multiple, long filaments. Floating form globular. Flagellate 19 to 29 (mean 24.2) μm long. Cyst with diameter 6 to 10 (mean 7.8) μm.

Type locality: Fuencaliente, La Palma Island, Canary Islands, Spain. 28° 27′ N 17° 50′ W.

Habitat: Saline and marine sediments.

Syntype: Protargol preparations of the culture FUEN4 with *M. disparata*, *Cyclidium* sp., and *Chilomastix cuspidata*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/1, 10/2 and 10/14.

Etymology: *disparata* (Latin adj.) separated, divided. The name points out the disproportionality between the flagellate and amoeba stage.

Monopylocystis robusta n. sp.

urn:lsid:zoobank.org:act:1BF60B25-172C-49E7-B5D8-A983F20DCDD6.

Diagnosis: *Monopylocystis* with locomotive amoeba larger than in other species, 18 to 51 (mean 32.2) μm long. Nucleus located in the anterior part of the granuloplasm and deformed during locomotion. Floating form globular. Flagellate unknown. Cyst with diameter of 9 to 14 (mean 12.2) μm.

Type locality: Fuencaliente, La Palma island, Canary Islands, Spain. 28° 27′ N 17° 50′ W.

Habitat: Saline sediments.

Syntype: Protargol preparations of the culture FUEN3 with *M. robusta* and an unidentified flagellate, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/3–10/5.

Etymology: *robusta* (Latin adj.) strong, robust (originally oaken). The amoeba of *M. robusta* n. sp. is the largest amoeba in the genus.

Monopylocystis elegans n. sp.

urn:lsid:zoobank.org:act:9E805F86-855B-4B91-BE4D-6DF46F19C29B.

Diagnosis: *Monopylocystis* with locomotive amoeba 16 to 51 (mean 23.2) μm long. Unique among *Monopylocystis* by the nucleus, which does not occupy a stable position in the granuloplasm and is not deformed during locomotion. Floating form globular. Flagellate 13 to 25 (mean 18.1) μm long. Cyst with diameter 7 to 11 (mean 9) μm.

Type locality: Evros delta, Greece. 40° 48′ N 26° 01′ W.

Habitat: Brackish sediments.

Syntype: Protargol preparations of the culture EVROS1M with *M. elegans* and an unidentified flagellate, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 9/98–9/100.

Etymology: *elegans* (Latin adj.) elegant, nice.

Genus *Harpagon* Pánek, Silberman, Yubuki, Leander & Cepicka 2012

Diagnosis: Freshwater and living in inland salt marshes. Uninucleate, tetrakont flagellates. Ventral groove of the flagellate up to 2/3 of the cell length. Amoeba and cyst unknown. Flagellates form several distinct morphotypes. Nucleus with several parietal nucleoli or with peripheral nucleolar layer beneath the nuclear membrane. Phylogenetically distinct from *Pseudoharpagon* Pánek, Silberman, Yubuki, Leander & Cepicka 2012.

Type species: *Tetramitus descissus* Perty 1852 by original designation.

Included species: *H. descissus* (Perty, 1852)=*Percolomonas descissus* (Perty, 1852)=*Tetramitus descissus* Perty1852; *H. schusteri* Pánek, Silberman, Yubuki, Leander & Cepicka 2012; *H. salinus* n. sp.

Harpagon salinus n. sp.

urn:lsid:zoobank.org:act:47F7D812-DBA6-443D-978B-576701584655.

Diagnosis: *Harpagon* living in inland salt marshes. Flagellates 11 to 17 (mean 13.7) μm long. Ventral groove 1/2 to 2/3 of the cell length. Amoeba and cyst unknown. Nucleolar material peripheral, distributed in a thin layer beneath the nuclear membrane.

Type locality: Einot Tsukim Nature Reserve, Israel. 31° 42′ N 35° 27′ E.

Habitat: Inland salt marsh sediments.

Syntype: Protargol preparations of the culture TSUKIM with *H. salinus* and *Monopylocystis visvasvarai*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/6–10/8 and 10/46–10/48.

Etymology: *salinus* (Latin adj.) of or belonging to salt. First member of the genus *Harpagon* isolated from a saline habitat.

Genus *Pseudoharpagon* Pánek, Silberman, Yubuki, Leander & Cepicka 2012

Diagnosis: Marine or brackish. Uninucleate. Flagellate, cyst and amoeba stages ancestrally present, one or two stages unknown in some species. Amoeba without clear demarcation between granuloplasm and hyaloplasm. Flagellate tetrakont to multiflagellate, with a ventral groove reaching up to 2/3 of the cell length. Nucleolar material peripheral, distributed in a thin layer beneath the nuclear membrane. Cysts rounded, with one or more (most often two) relatively long projections. Phylogenetically distinct from *Harpagon* Pánek, Silberman, Yubuki, Leander & Cepicka 2012.

Type species: *Pseudoharpagon pertyi* Pánek, Silberman, Yubuki, Leander & Cepicka 2012 by monotypy.

Included species: *Pseudoharpagon pertyi* Pánek, Silberman, Yubuki, Leander & Cepicka 2012; *Pseudoharpagon longus* n. sp.; *Pseudoharpagon tertius* n. sp.

Pseudoharpagon longus n. sp.

urn:lsid:zoobank.org:act:0436A8A6-0506-4618-B7C1-82EC0B859545.

Diagnosis: *Pseudoharpagon* with locomotive amoeba 29 to 56 (mean 38.5) μm long. Flagellate 14 to 35 (mean 20.4) μm long, predominantly with five flagella. Quadriflagellate and multiflagellate cells present. Cyst with diameter 16 to 22 (mean 19) μm.

Type locality: Evros delta, Greece. 40° 48′ N 26° 01′ E.

Habitat: Brackish sediments.

Syntype: Protargol preparations of the culture EVROS1I with *Pseudoharpagon longus* and *Andalucia incarcerata*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 10/9–10/11.

Etymology: *longus* (Latin adj.) long. Flagellates of *Pseudoharpagon longus* are longer and more elongated than those of the other *Pseudoharpagon* species.

Pseudoharpagon tertius n. sp.

urn:lsid:zoobank.org:act:7C3F220C-032D-44F8-B9C0-063E5DEC7B49.

Diagnosis: *Pseudoharpagon* with flagellate 10 to 22 (mean 15.5) μm long. Quadriflagellate. Amoeba stage present. Cysts unknown. Phylogenetically distinct from *Pseudoharpagon pertyi*.

Type locality: Porto Lago, Greece. 41° 00′ N 25° 06′ E.

Habitat: Marine sediments.

Syntype: Protargol preparations of the culture LAGOS1P with *Pseudoharpagon tertius*, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 8/90–8/92.

Etymology: *tertius* (Latin adj.) third. *Pseudoharpagon tertius* is the third described species of *Pseudoharpagon*.

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5.3. Pánek *et al.* 2014b

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Creneis carolina gen. et sp. nov. (Heterolobosea), a Novel Marine Anaerobic Protist with Strikingly Derived Morphology and Life Cycle

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We report the light-microscopic morphology and ultrastructure of a novel free-living, heterotrophic protist, *Creneis carolina* gen. et sp. nov. isolated from marine anoxic sediments. *C. carolina* is a heterotrophic, obligately anaerobic amoeboid flagellate, and superficially resembles *Mastigamoeba* (Amoebozoa: Archamoebae) or *Breviata* (Breviatea) by possessing a single anterior flagellum closely associated with the nucleus, and because it appears to be an anaerobe. However, its life cycle contains multiflagellate cells with an unusual morphology. The structure of the mastigont of *C. carolina* is unique and not readily comparable with any eukaryotic group. Unexpectedly, phylogenetic analyses of SSU rDNA and of a concatenate of α- and β-tubulin genes with SSU rDNA convincingly showed that *C. carolina* is a member of Heterolobosea and belongs to the taxon Tetramitia.

Key words: Heterolobosea; anaerobiosis; ultrastructure; new species; evolution; molecular phylogeny.

Introduction

Although most described species of eukaryotic organisms are multicellular, the overwhelming majority of high-level lineages of eukaryotes consist exclusively or almost exclusively of unicellular protists (Adl et al. 2012). Much of the true diversity of protists remains to be described, even at the level of major lineages (Kolisko et al. 2010; Pawlowski et al. 2012). This is demonstrated by the many recent discoveries of protists with extraordinary morphology and ultrastructure that often represented novel deep lineages (e.g. Breglia et al. 2010; Moore et al. 2008; Yabuki et al. 2010, 2011, 2013; Yamaguchi et al. 2012). It is further confirmed by numerous sequence-based surveys of eukaryotic microbial diversity that have revealed a huge undescribed diversity of protists (e.g. Massana and Pedrós-Alió 2008; Massana et al. 2004; Stoeck et al. 2003). The low abundances of most OTUs in sequence libraries indicates that most taxa are relatively rare

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in the environment and constitute a rare biosphere (Caron and Countway 2009; Countway et al. 2007). The evolutionary history of protists is closely connected to the evolution of the eukaryotic cell itself. Therefore, knowledge about protistan diversity and evolution, including discovery and subsequent morphological, ultrastructural, ecological and genomic characterization of novel protistan lineages, is crucial for understanding the history of eukaryotic evolution as well as the underlying processes that direct it (Dawson and Paredez 2013; O'Malley et al. 2013).

Heterolobosea is a major eukaryotic group belonging to the supergroup Excavata (Adl et al. 2005; Hampl et al. 2009; Simpson and Roger 2004) that includes approximately 140 described species only (Pánek and Čepička 2012). The diversity of Heterolobosea as a group had been understudied until recently, particularly because research interest was focused primarily on the medically important genus *Naegleria*, which currently contains around 30 % of heteroloboseid species (Guzmán-Fierros et al. 2008).

Many heteroloboseids are amoeboflagellates with three morphologically and ecologically distinct life history stages; amoebae, flagellates and cysts (see Patterson et al. 2000). The existence of these stages and the alternation between them is most likely ancestral to the whole taxon Heterolobosea (Harding et al. 2013). During the process known as amoeba-to-flagellate transformation the flagellar apparatus, including basal bodies, is assembled de novo in the amoeba cell. The process has been studied in detail in *Naegleria gruberi*, where it is completed in only two hours (Lee 2010; Walsh 2007). Many heteroloboseids have probably completely lost the flagellate or amoeba stage and exist as pure amoebae or flagellates. Members of Acrasidae, one of the heteroloboseid lineages, have developed a simple form of multicellularity and are the only known multicellular excavates (Brown et al. 2012). Another lineage, *Stephanopogon*, comprises multiflagellate protists that are convergent with ciliates (Corliss 1979; Entz 1884). Ultrastructural data, particularly the presence of discoidal mitochondrial cristae and an absence of many typical ciliate features, indicated that the resemblance was only superficial (Lipscomb and Corliss 1982; Patterson and Brugerolle 1988), and a close relationship between *Stephanopogon* and Euglenozoa and/or Heterolobosea was proposed (Cavalier-Smith 1981, 1991; Patterson and Brugerolle 1988). The precise phylogenetic position of *Stephanopogon* has been definitively elucidated only recently, using molecular phylogenetics (Cavalier-Smith and Nikolaev 2008; Yubuki and Leander 2008).

Heteroloboseids are heterotrophic and predominantly free-living (see Pánek and Čepička 2012), although a few species are endobionts of animals and several members of *Naegleria*, as well as *Paravahlkampfia francinae* are facultative pathogens of animals and humans (De Jonckheere 2002; Visvesvara et al. 2007, 2009). Heteroloboseid species have been recorded from various habitats – they are mostly marine and freshwater or live in soils, but they have also been found in hypersaline brines, extremely acidic waters, anaerobic sediments or thermal springs (e.g. Baumgartner et al. 2009; De Jonckheere et al. 2009, 2011a, 2011b; Garstecki et al. 2005; Murtagh et al. 2002; Pánek et al. 2012; Park et al. 2007, 2009; Park and Simpson 2011). SSU rDNA phylogenies show that Heterolobosea very probably spread into most of these extreme environments more than once (Pánek et al. 2012; Park and Simpson 2011; Park et al. 2012). By contrast, almost all described anaerobic species of Heterolobosea form a single clade and are classified into the family Psalteriomonadidae (Pánek et al. 2012), with *Pleurostomum flabellatum* being a possible (but very uncertain) exception (Park et al. 2007).

Here we report the discovery of a novel free-living anaerobic organism, *Creneis carolina* gen. et sp. nov., that was not classifiable into any major group of eukaryotes solely on the basis of morphology and cell structure. A relationship of *C. carolina* with tetramitid Heterolobosea was revealed only by molecular phylogenetic analyses. This new species has a unique life cycle, and a morphology that is unlike that of other heteroloboseids, and its precise phylogenetic position within Heterolobosea remains unclear.

Results

Light-Microscopy

The life cycle of *Creneis carolina* gen. et sp. nov. is polymorphic and contains several distinct forms (Figs 1–4). The main trophic stage in the culture is represented by an amoeboid flagellate with a single anterior flagellum (Fig. 1A – C). These cells are on average 20.3 μm long (S.D. ± 4.0; range 14.0 – 31.7) and 12.8 μm wide (S.D. ± 4.6; range 7.8 – 30.2), with the average length/width ratio being 1.7 (n = 30, live cells). They primarily move using amoeboid locomotion by forming a broad anterior pseudopodium that is hyaloplasmic

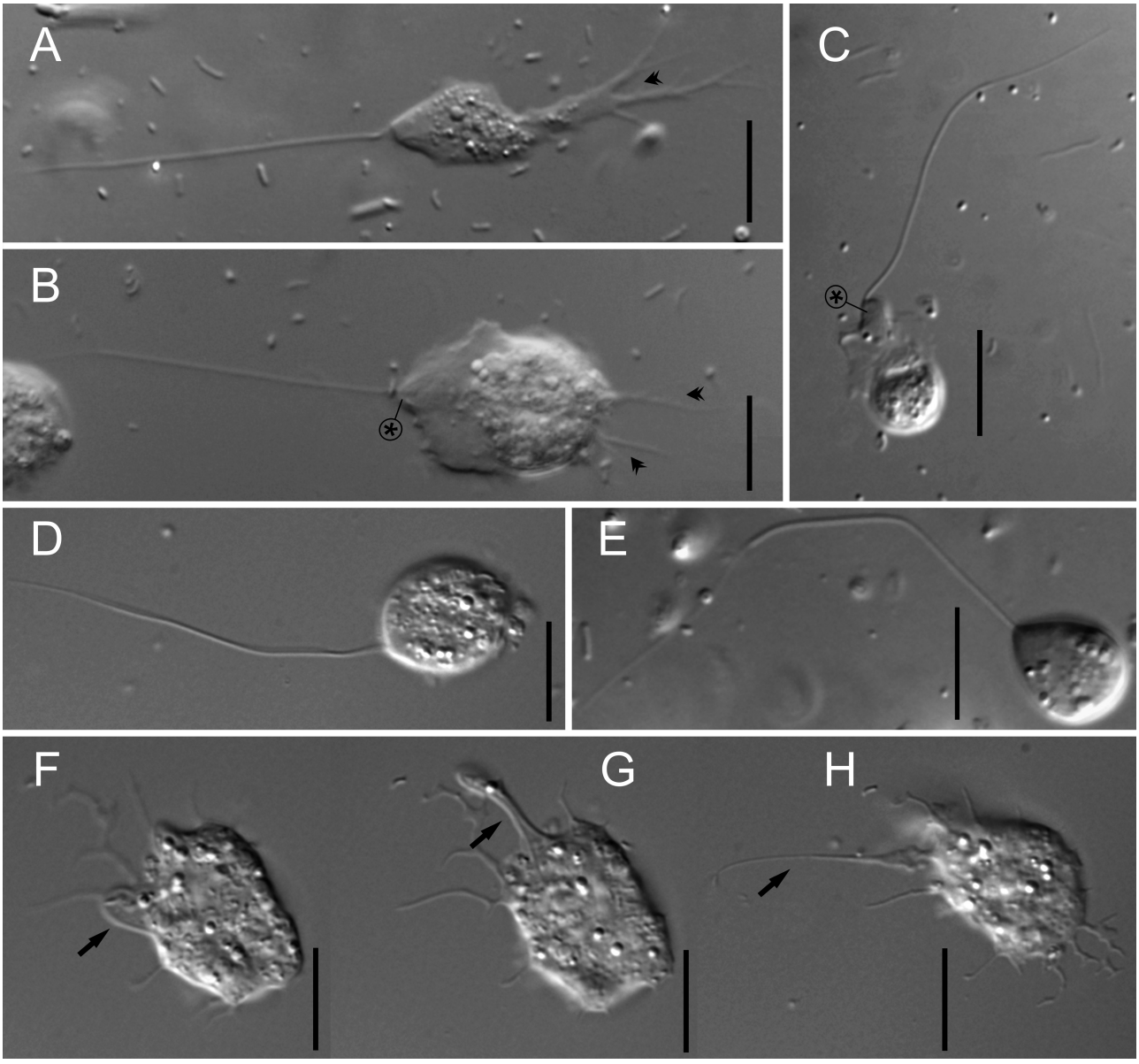

Figure 1. Living *Creneis carolina* amoeboid flagellates with a single anterior flagellum (the most abundant trophic stage in culture). Differential interference contrast. **A – C.** Cells undergoing amoeboid locomotion. **D, E.** Floating forms without pseudopodia. **F – H.** Internalization and externalization of the flagellum of a single cell observed over 3 minutes. Labels: arrow – flagellum; double arrowhead – uroidal filaments; circle with asterisk – nucleus. Scale bars: 10 μm.

and extremely flattened, but non-eruptive (Fig. 1B, C). The rate of locomotion over a glass substrate at room temperature is 11 – 19 $\mu m.min^{-1}$ (n = 10). The posterior end of the locomoting cell usually produces a mass of adhesive filaments that are up to 40 μm in length and sometimes branched (Fig. 1A, B). The anterior pole of the hyaloplasm contains a protruding apical bulge that includes the nucleus and the site of flagellar insertion (Fig. 1B, C and Supplementary Material Fig. S1A). The nucleus is elongated, curved and somewhat irregular (Fig. 2A, C, F), and averages 5.6 μm long (S.D. ± 1.1; range 4.0 – 7.7) and 2.4 (S.D. ± 0.5; range 1.6 – 3.5) μm wide (n = 10, DAPI stained cells), It is usually close to one side of the cell, indicating a position near the cell membrane (Figs 2A, C, F, and Supplementary Material Fig. S1).

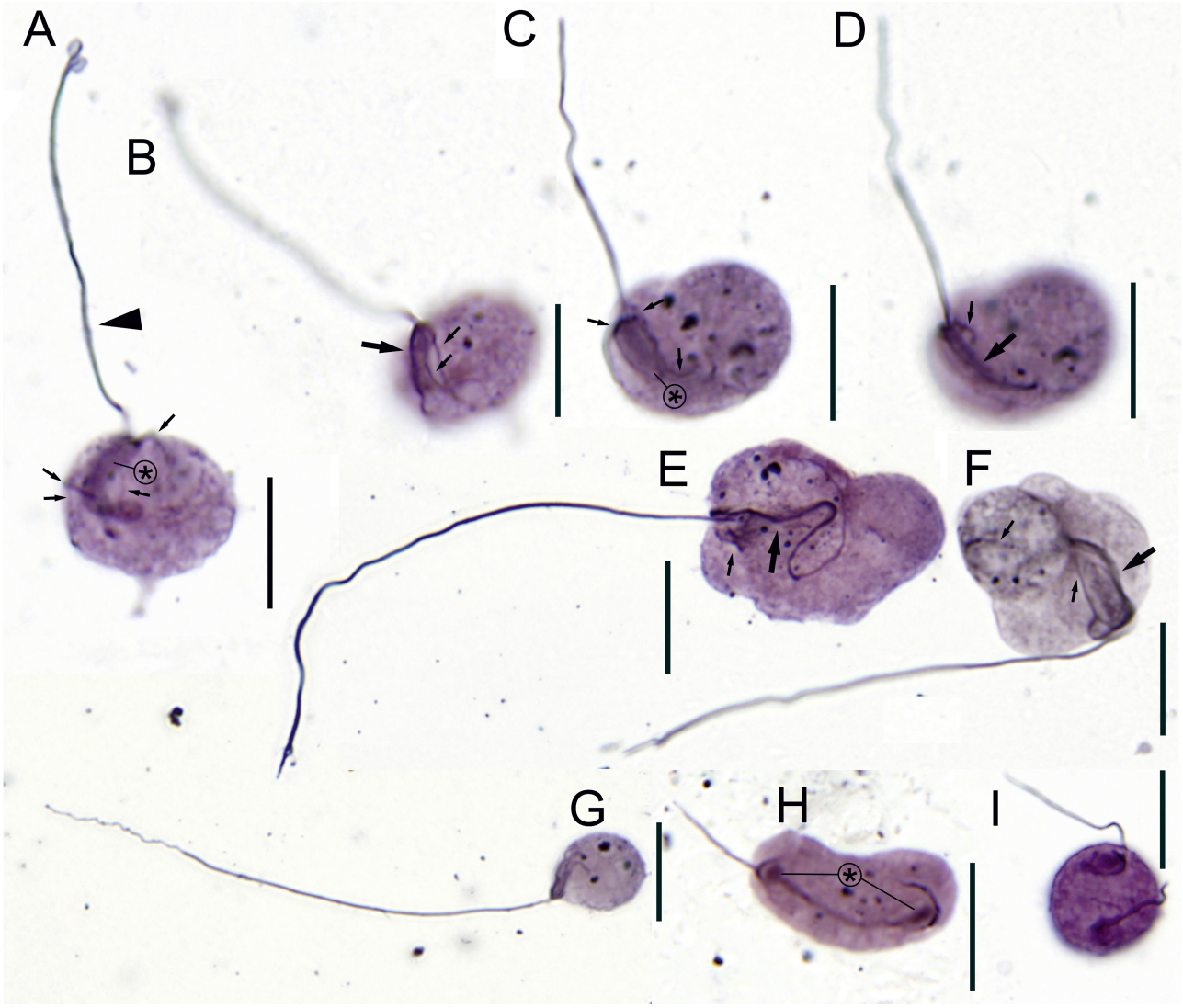

Figure 2. Protargol-stained uniflagellated and dividing cells of *Creneis carolina*. Bright-field micrographs. **A – F.** Amoeboflagellate cells with a single flagellum (C and D represent two different planes of the same cell). **G.** Rounded form. **H.** Cell during mitosis. **I.** Dividing cell with two nuclei and flagella. Labels: arrowhead – flagellum; circle with asterisk – nucleus; thick arrow – thick cytoskeletal structures; thin arrow – thin cytoskeletal structures. Scale bars: 10 μm.

The single flagellum is 43.2 μm long (± 4.8; 34.8 – 51.3; n = 15, live cells) and usually acronematic (Figs 1E, 2G). The flagellar base is closely associated with the anterior part of the nucleus and is most clearly visible in protargol-stained cells (Figs 2, 3E, F). During locomotion the flagellum is directed anteriorly (Fig. 1A, B), but beats very slowly and does not seem to have a significant effect on cell movement. The flagellum usually terminates with an acroneme of variable length (Figs 1C – E, 2G). The tip appears looped in some protargol-stained cells, which is probably a fixation artifact (Fig. 2A). Internalization or externalization of the entire flagellum over a period of minutes was repeatedly observed in living cells (Fig. 1F – H).

Amoeboid flagellates may take on a rounded form without pseudopodia (Fig. 1D – E). The flagellum of the rounded cells does not beat faster than that of crawling cells and it seems that these cells do not move actively. The rounded flagellate form averages 10.8 μm long (± 2.0; 6.8 – 14.8) and 8.2 μm wide (± 1.1; 6.3 – 10.1) with an average length/width ratio of 1.3 (n = 30, live cells).

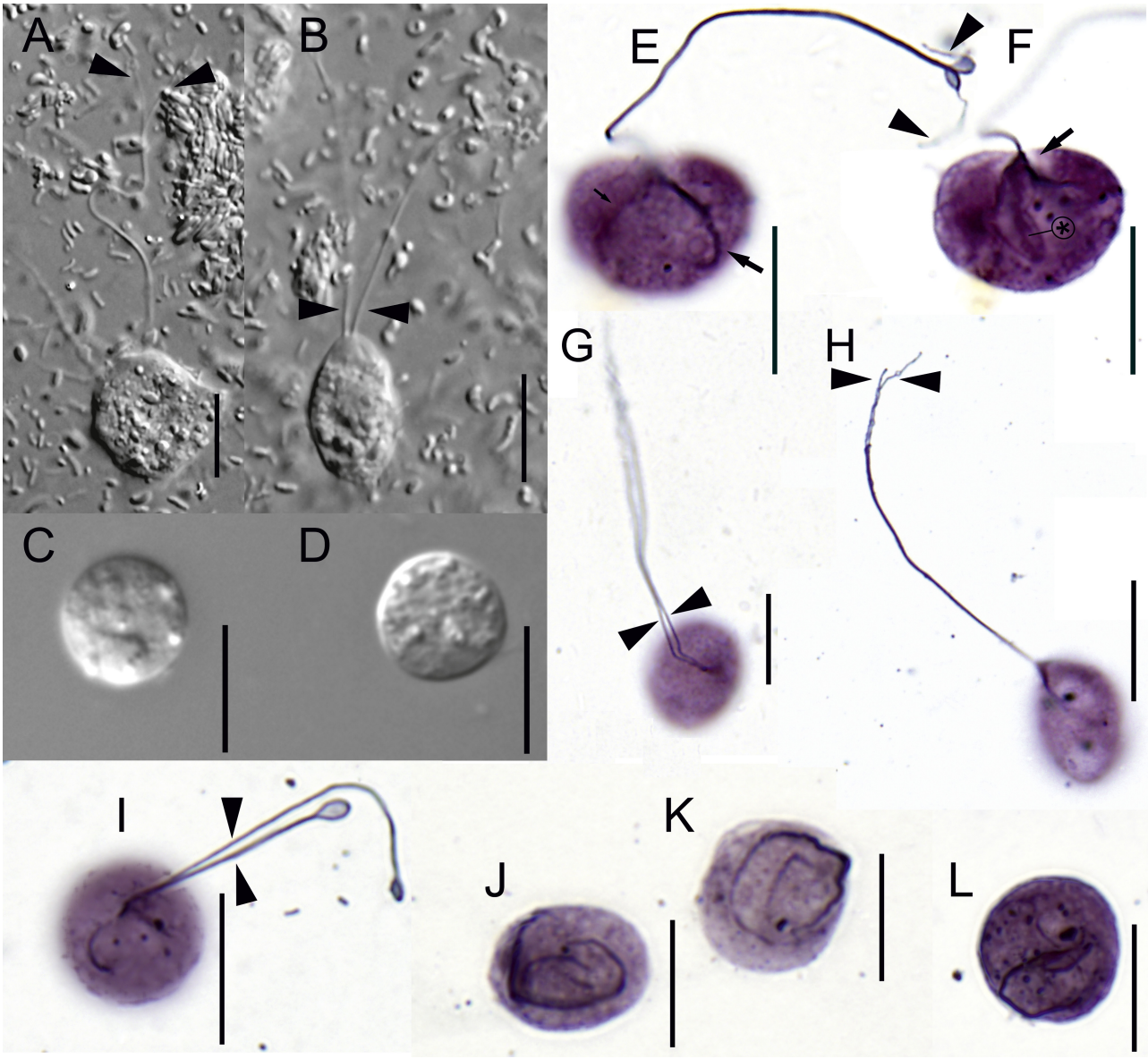

Figure 3. Biflagellated cells and 'resting forms' of *Creneis carolina*. **A, B.** Living biflagellated cells. Differential interference contrast. **C, D.** Living resting cells. Differential interference contrast. **E – I.** Protargol-stained biflagellated cells (E and F represent different planes of the same cell). Bright-field micrographs. **J – L.** Protargol-stained resting forms. Bright-field micrographs. Labels: arrowhead – flagella; circle with asterisk – nucleus; thick arrow – thick cytoskeletal structures; thin arrow – thin cytoskeletal structures. Scale bars: 10 μm.

A minority (<5%) of both amoeboid and rounded forms are biflagellated (Fig. 3). The flagella are equal in length and arise at the apex of the cell. The flagella are clearly separated in some biflagellate cells (Fig. 3B, G, I), whereas in others they are adpressed for most of their length and can be distinguished only with difficulty (Fig. 3A, E, H). Slow externalization of an entire second flagellum at the base of the first flagellum was documented in a single cell (Supplementary Material Fig. S2), and took approximately 30 mins. These biflagellate cells do not show any signs of division.

Two dividing cells were also observed (Fig. 2H, I). The first one was undergoing karyokinesis (Fig. 2H). It possessed a single short flagellum associated with one pole of the dividing nucleus. The second pole of the nucleus was associated with a dense fibre. The identity of the fibre remains uncertain, though it might have been an internalized or newly formed flagellum. The second cell

was binucleated and had two short flagella, each being associated with a daughter nucleus (Fig. 2I). Sexual reproduction was not observed.

Globular, aflagellated nonmotile cells ("resting forms") are also formed (Fig. 3C – D, 3J – L). These are 10.9 μm long (± 1.0; 8.7 – 12.0) and 10.4 μm wide (± 0.9; 8.6 – 11.7; n = 15, live cells).

Protargol staining revealed an intricate cytoskeletal system in the amoeboid flagellates (Figs 2, 3). It consists of several fibers that associate with the nucleus and/or flagellar base in the anterior part of the cell. One of these fibres, the 'thick fiber', is relatively long, with both ends running to the posterior part of the cell, along variable circuitous paths (Fig. 2D – F). This fibre is thickest in the anterior part of the cell, where it runs directly under the cell surface. This 'thick fiber' corresponds to the 'F+R2a' complex observed by TEM (see below). The aflagellated resting cells contain a similar system of fibers, possibly augmented by an internalized flagellum appearing as an additional thick protargol-stained element (Fig. 3J – L).

The rarest morphotype is an elongate, fast-swimming, multiflagellate cell with approximately 14 flagella (Fig. 4). Only ca. 50 of these cells have been observed during several years of cultivation. These cells were usually 20 – 25 μm long and 8 – 9 μm wide when live, and often had a tapered end (Fig. 4C, D), although more rounded cells were also observed (Fig. 4A, B are moribund multiflagellate cells). A few cells were observed that had the posterior end divided to two or three tapering projections (not shown). Two flagella arose at the apex of the cell and were directed anteriorly. The remaining flagella inserted laterally in the equatorial region of the cell and were directed postero-laterally. Protargol-stained multiflagellates possessed neither visible pseudopodia nor cytostome, but the nucleus displayed a similar structure to that of the amoeboflagellate and resting stages (Fig. 4E – H). The multiflagellate stage was apparently transient and short-lived under culture conditions.

Transmission Electron Microscopy

The cell structure of the typical amoeboid flagellate stage of *Creneis carolina* (with a single anterior flagellum) was examined by TEM. The general terminology for ultrastructural elements was adopted from Yubuki and Leander (2013). The 'left' and 'right' sides of the cell were defined on the assumption that the flagellum represents the anterior end of the cell, and that 'R2a' (see below) extends along the 'ventral' face.

The nucleus is elongated and usually lies on the right/dorsal side of the cell (Fig. 5A-B). It usually has a conspicuous depression 1/3rd of the way along its anteroposterior axis (Fig. 5B) and possesses a central, spherical nucleolus (Fig. 9A). Cells do not possess a focussed Golgi apparatus represented by stacked membrane-bounded sacs. There are many mitochondrion-related organelles (MROs) per cell; they are rounded, 300 – 500 nm in diameter, and bounded by two membranes (M in Fig. 5C, 9F). No clear examples of cristae were observed (in ~100 cells, from five separate fixations). Since the inner mitochondrial membrane is often well preserved, it is unlikely that the absence of cristae is a fixation artifact. The MROs are not found in close association with endoplasmic reticulum. Some cells contain lipid droplets up to 1.4 μm in diameter (Fig. 5A). Cells typically contain numerous food vacuoles with bacteria (Fig. 5B). Fine pseudopodia with relatively dense interiors project from the cell in various positions (Fig. 5D).

The flagellar apparatus is closely associated with one pole of the nucleus at the apex of the cell (Fig. 5B). It is composed of a single, unpaired basal body bearing a flagellum, three main microtubular structures (putative R1, split R2 and the ribbon-like 'F'), and one noteworthy non-microtubular fibre, a rhizoplast (Fig. 6C). We never observed "barren" basal bodies.

The axoneme of the flagellum displays the typical '9+2' arrangement of microtubules and bears no vanes or paraxonemal structures (Fig. 5B, E – G). The central pair of axonemal microtubules originates in a conspicuous electron-dense globule that sits immediately above the transitional plate (Figs 5E, 7D). The basal body is 350 – 400 nm long, with the proximal end connected by a very short striated element to a 'pad', or fibrous sheet, that in turn is closely connected to the nuclear envelope (Figs 5E, 6A, 7B, C). We observed naked axonemes within the cell cytoplasm, usually immediately below the cell membrane (Fig. 5F), and also entire flagella located within membrane-delimited tunnels (Fig. 5G). Either or both of these forms could be related to the reversible flagellar internalization process observed in living cells by light microscopy (e.g. Fig. 1G; see above), but it is also possible that the former is a fixation artifact.

The microtubular band that is tentatively labeled 'R1' originates in the vicinity of the anterior side of the basal body and roughly orthogonally to it (Figs 6A, 7A – D). It heads left, running near the nucleus and is composed of approximately eight microtubules at its broadest (Figs 8B – E, 9C,

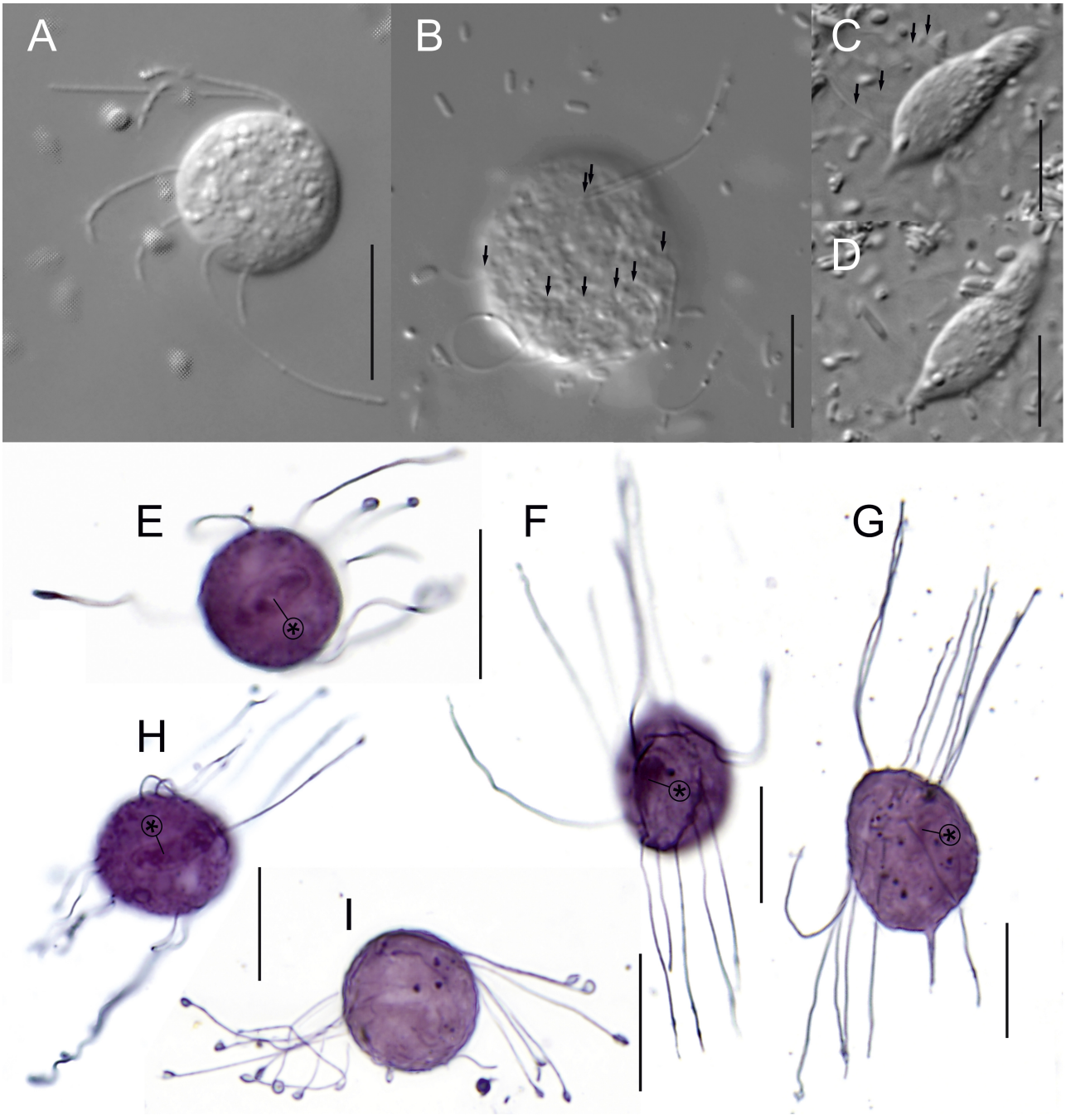

Figure 4. Multiflagellated forms of *Creneis carolina*. **A, B.** Moribund cells. Differential interference contrast. **C, D.** Living cells with typical morphology. Differential interference contrast. **E – I.** Protargol-stained cells. Bright-field micrographs. Labels: arrow – flagellum; circle with asterisk – nucleus. Scale bars: 10 μm.

9F). R2 originates as a single long row near the posterior-left side of the basal body, at an oblique angle to it (Figs 6A – E, 8C). R2 immediately splits into two distinct parts – 'R2a' and 'R2b', where R2a is closer to the basal body (Figs 7A – D, 8C – E). R2b heads left and curves closely around the nucleus, running quite close to the putative R1 (Figs 8C – E, 9A, F). R2a initially consists of a curved ribbon of ~10 microtubules, with its concave side facing the cell surface. The concave side of 'R2a' displays a layer of spoked connecting material, at least near its origin (Figs 6A, B, 7A – C).

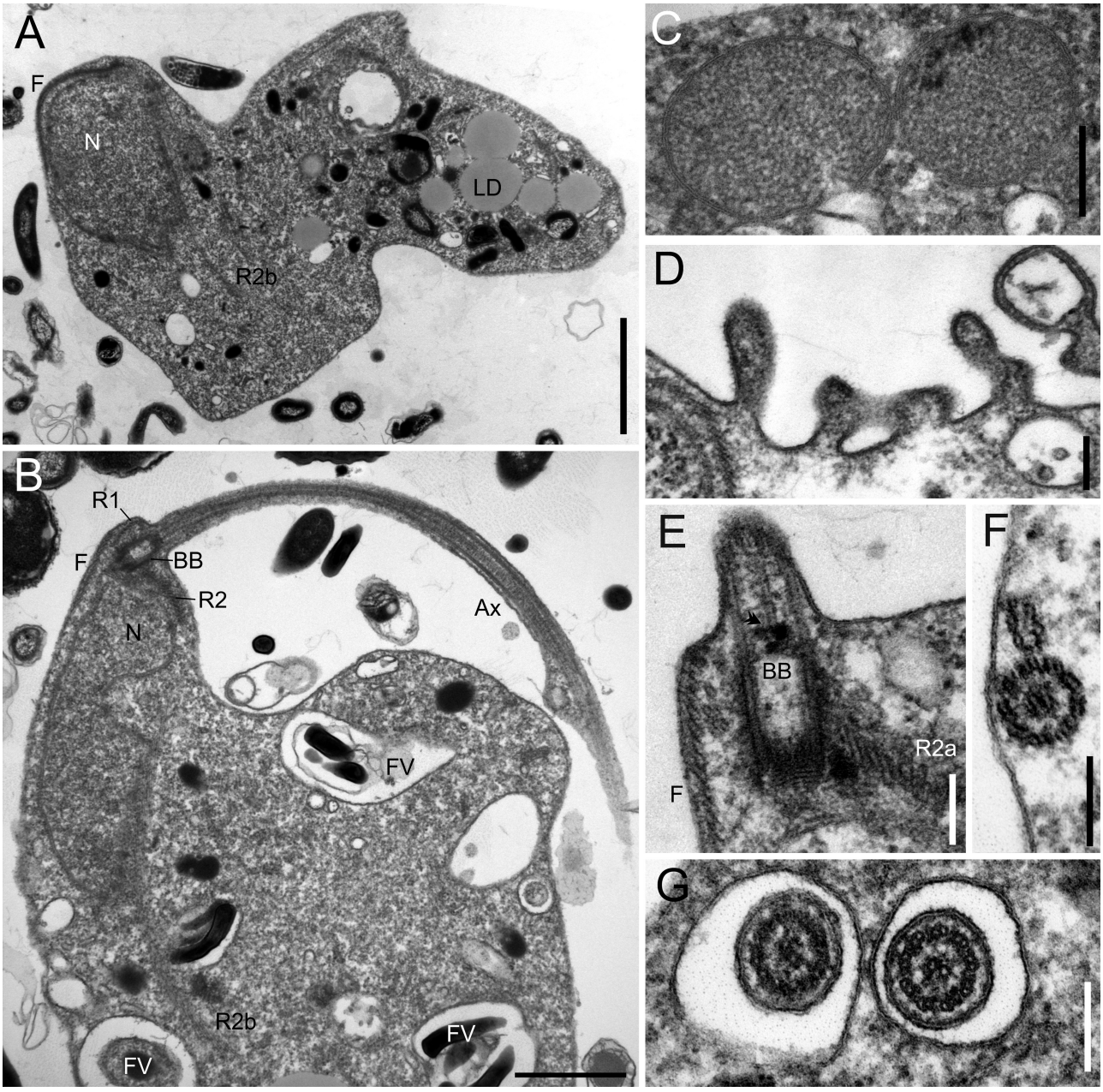

Figure 5. Transmission electron micrographs of *Creneis carolina*. **A.** Longitudinal section through the cell. **B.** Longitudinal section through the anterior part of the cell showing the flagellar apparatus apressed to the nucleus. **C.** Mitochondrion-related organelles lacking cristae. **D.** Fine pseudopodia. **E.** Longitudinal section through the basal body of the single flagellum showing ball-like globule near the transitional plate. **F.** Naked axoneme within the cell cytoplasm (particular life stage uncertain). **G.** Flagellum or flagella located in the cytoplasm within membrane-bounded tunnels. Abbreviations: Ax – axoneme of the flagellum; BB – basal body of the flagellum; F – 'F' element; FV – food vacuole; LD – lipid droplets; N – nucleus; R1 – putative microtubular root R1; R2 – microtubular root R2; R2a – the first part of split R2 closer to the basal body; R2b – the second part of split R2; double-arrowhead – ball-like globule near the transitional plate of the flagellum. Scale bars: 2 μm for A; 1 μm for B; 200 nm for C – G.

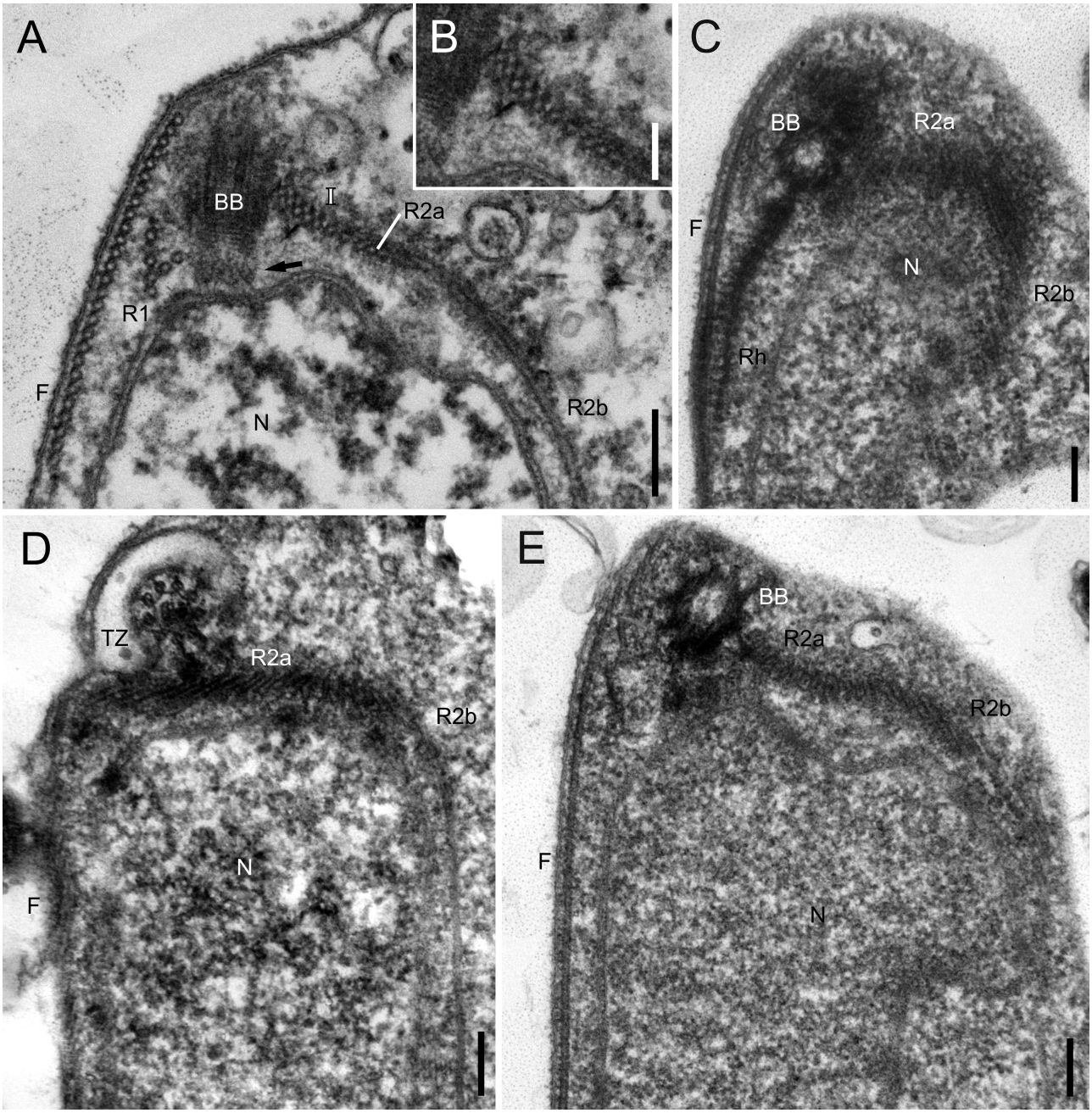

Figure 6. Transmission electron micrographs of *Creneis carolina* – the flagellar apparatus (sections in different orientations). **A.** Section through the flagellar apparatus showing main microtubular components. **B**. Detail of the I fibre. **C.** Section through the flagellar apparatus approximately orthogonal to (A), showing rhizoplast. **D.** Section through the flagellar apparatus with transition zone in transverse section, setting absolute orientation based on the axoneme (tip to base orientation). **E.** Section through the flagellar apparatus showing extent of the 'F' element. Abbreviations: BB – basal body of the flagellum; F – 'F' element; I – I fibre; N – nucleus; Rh –rhizoplast; R1 – putative microtubular root R1; R2 – microtubular root R2; R2a – the first part of split R2 closer to the basal body, R2b – the second part of split R2; TZ – transition zone. Scale bars: 200 nm (A, C, D, E), 100 nm (B).

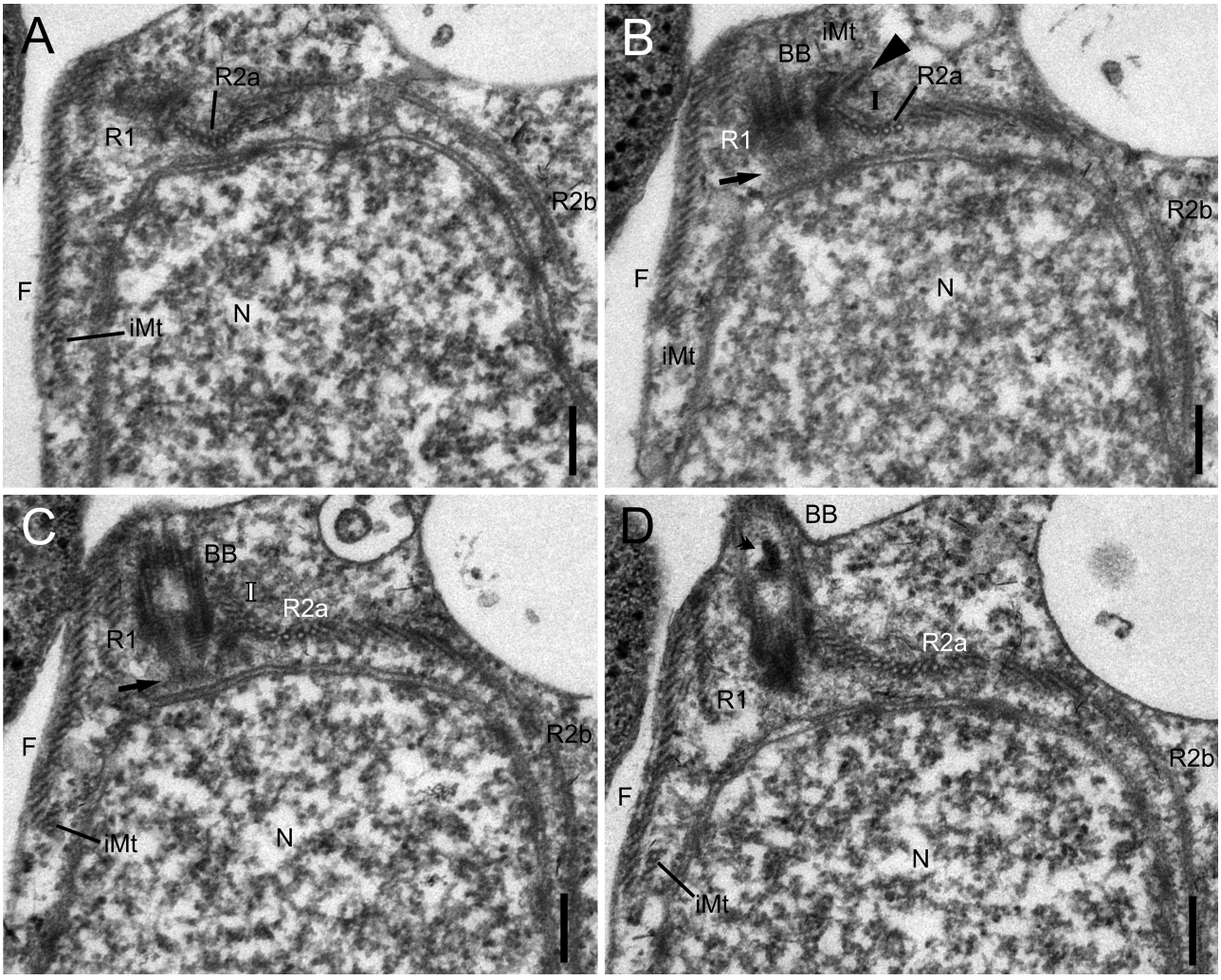

Figure 7. Transmission electron micrographs of *Creneis carolina* – the flagellar apparatus. **A – D.** Non-consecutive serial sections through the flagellar apparatus, showing start of the R2. Note curved shape of R2a and supporting material (probable I fibre). Abbreviations: BB – basal body of the flagellum; F – 'F' element; I – I fibre; iMt – individual or small groups of microtubules that originate close to the basal body between the 'F' and the nucleus; N – nucleus; R1 – putative microtubular root R1; R2 – microtubular root R2; R2a – the first part of split R2 closer to the basal body; R2b – the second part of split R2; arrow – 'pad-like' structure between the basal body and nucleus; arrowhead – 'double-leaved spur' structure near the basal body and the convex side of the 'R2a' near its origin; double-arrowhead – ball-like globule near the transitional plate of the flagellum. Scale bars: 200 nm.

There is also a small double-leaved 'spur' structure that projects from the angle between the basal body and the concave side of R2a (Fig. 7A, B). The former might represent the 'I' fibre – see discussion for further comments. The convex face is associated with more indistinct material that includes diffuse connections to the basal body and a 'pad' (Figs 6A, 7B, C).

After its origin, R2a heads right and curves ventrally, running under the cell membrane, and connects with the 'F' element (see below) to form a single massive microtubular complex with a L- or V- shaped cross-section (Figs 8E, F, 9A – C). This corresponds to the "thick fibre" that is visible in protargol-stained cells (see discussion for details). The 'inner' side (formerly the convex face) of R2a develops a conspicuous set of thick, forked spokes (Fig. 8F). There is also a striated non-microtubular fibre (rhizoplast) that originates near the basal body, then runs under the right edge of R2a, near its junction with F (Figs 6C, 8D – F). A smaller dense fibre 'X' lies immediately under the cell membrane and

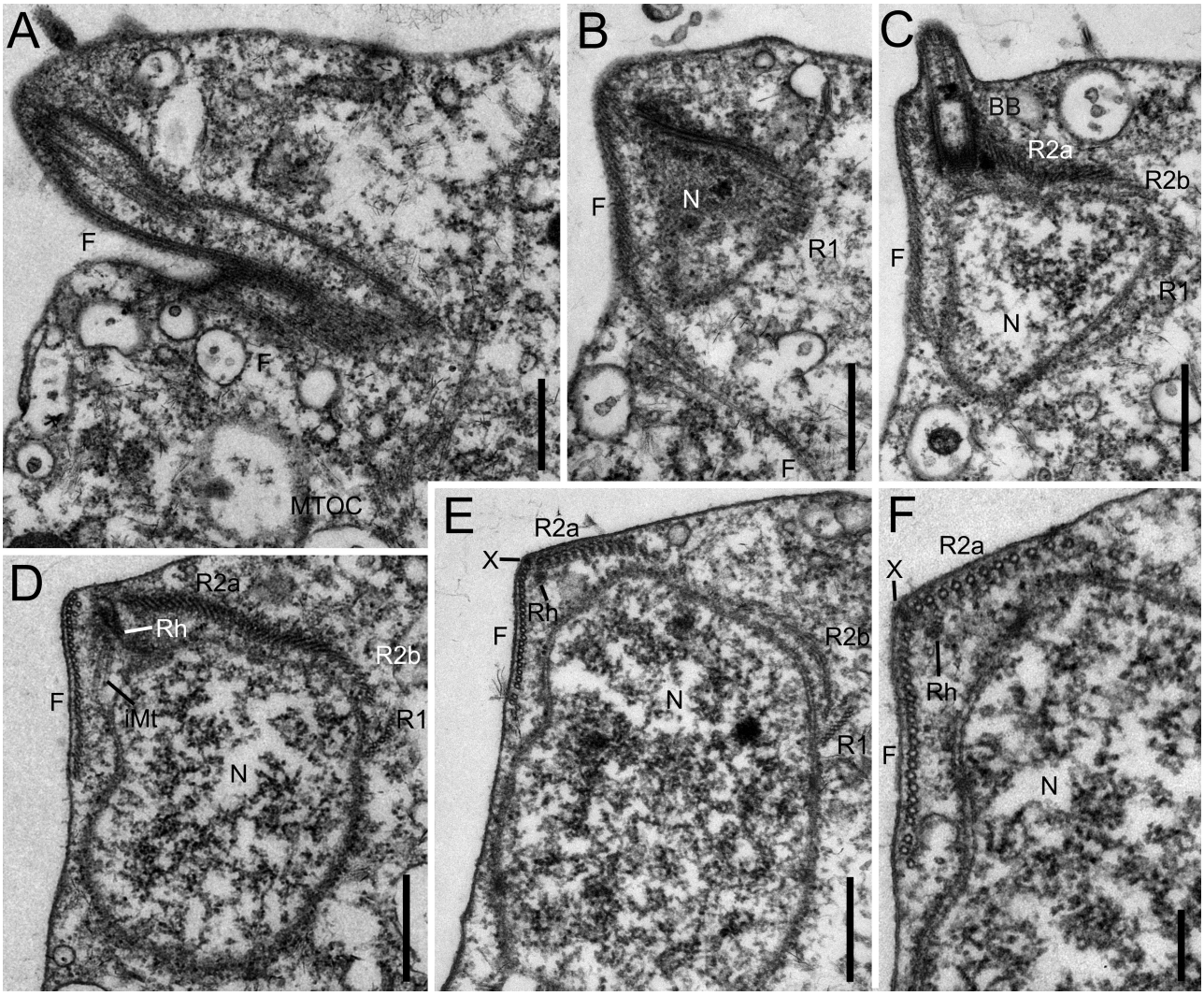

Figure 8. Transmission electron micrographs of *Creneis carolina* – microtubular structures near the flagellar apparatus (non-consecutive serial sections). **A.** Section through the flagellar apparatus immediately anterior/left of the nucleus showing left arm of the F element and associated MTOC-like region. **B.** Section through the flagellar apparatus showing course of R1 along left side of the nucleus. **C.** Section through the flagellar apparatus showing split of R2, with 'R1' continuing along the left side of the nucleus. **D.** Section immediately posterior/right of basal body showing the right arm of the F element, origin of rhizoplast, and individual microtubules between the 'F' element and the nucleus and rhizoplast. **E.** Section showing complete separation of R2a and R2b, and connection of the 'F' element and 'R2a'. **F.** Detail of associated 'F' fibre and 'R2a' showing conspicuous set of thick, forked spokes. Note also positions of rhizoplast and 'X' element. Abbreviations: BB – basal body of the flagellum; F – 'F' element; iMt – individual microtubules that originate close to the basal body between the 'F' and the nucleus; MTOC – microtubule-organizing centre-like region near the flagellar apparatus; N – nucleus; Rh – rhizoplast; R1 – putative microtubular root R1; R2 – microtubular root R2; R2a – the first part of split R2 closer to the basal body; R2b – the second part of split R2; X – X element. Scale bars: 500 nm for A – E; 200 nm for F.

defines the junction between R2a and F (Fig. 8E, F).

The F group of >20 linked microtubules does not originate near the flagellar basal body, but instead travels past the anterior side of the basal body as a hood (Fig. 8A – E). On the left side it runs deeply into the cell (Fig. 9C). On the right side it continues immediately beneath the cell membrane alongside R2a (Figs 6A – E, 8C – F, 9A – C). The F+R2a group (without the rhizoplast or spokes) continues along

the cell surface beyond the region of the nucleus (Fig. 9B), but its precise end-point was not determined.

We observed a number of ribbons composed of several microtubules running through the cell (e.g. Fig. 9C – G). Some of them that are near the nucleus are actually continuations of parts or all of R1, R2b and the left arm of F (Fig. 9A, F), while others originate in a MTOC-like region associated with the 'left' arm of F on the other side of the nucleus from the flagellum (Figs 8A, 9C – F). There are also many individual microtubules or small groups of microtubules that originate close to the basal body, between the 'F' element and the nucleus (Figs 7A – D, 8D). We also observed a diffuse microfibrillar bundle in at least one cell, which was only in loose connection with the major microtubular elements of the cytoskeleton (Fig. 9A).

An interpretative diagram of the flagellar apparatus is shown in Figure 10 and a comparison between uniflagellate cell of *Creneis* and flagellates from related protistan groups is shown in Table 1.

Phylogenetic Analyses

Small subunit (SSU) rRNA gene sequences and inferred α- and β-tubulin amino acid sequences were used to infer the relationships of *Creneis* within eukaryotes. Both the phylogeny of SSU rRNA genes (Fig. 11) and the concatenation of the SSU rRNA gene with α- and β-tubulin proteins (Fig. 13) showed that *Creneis* forms a very long branch but is robustly placed inside Heterolobosea (bootstrap support $\geq$ 97%, posterior probability > 0.97). In the analyses based on SSU rRNA gene sequences (Figs 11, 12), *Creneis* was robustly placed within the largest heteroloboseid subgroup, Tetramitia, with bootstrap support $\geq$ 96%, and posterior probability > 0.97. Pinpointing the position of *Creneis* inside Tetramitia was difficult. Depending on the method of tree construction and the data set, the SSU rRNA gene analyses placed *Creneis* either as sister to Psalteriomonadidae (maximum likelihood analysis of the broad SSU rRNA data set; Fig. 11), or as sister to *Percolomonas*+*Stephanopogon* (maximum likelihood analysis of the Heterolobosea SSU rRNA data set; Fig. 12), or as a sister to *Stephanopogon* itself (Bayesian analysis of the broad SSU rRNA data set; Fig. 11). The placement of *Creneis* was never robustly supported in these analyses, with the weak exception of the PhyloBayes analysis of the broader SSU rRNA data set (Fig. 11), which placed *Creneis* within the *Percolomonas*+*Stephanopogon* clade with posterior probability 0.93.

The concatenated-gene data set contained a much lower diversity of Heterolobosea. Nonetheless it did include representatives of both potential sister groups to *Creneis* identified above, namely *Percolomonas*+*Stephanopogon*, and Psalteriomonadidae (*Pseudoharpagon* and *Harpagon*), albeit β-tubulin sequences were lacking for *Pseudoharpagon* and *Harpagon*. In analyses of the concatenated data set, a sister-group relationship between *Creneis* and Psalteriomonadidae was recovered and was moderately supported (bootstrap support 72%, posterior probability 0.99; Fig. 13).

Secondary Structure of the SSU rRNA Molecule

The secondary structure of the V3 variable region of the SSU rRNA molecule of *Creneis carolina* was examined in order to determine whether the Tetramitia-specific helix 17_1 was present (see Harding et al. 2013; Nikolaev et al. 2004; Park and Simpson 2011; Park et al. 2012; Wuyts et al. 2001). The sequence corresponding to V3 was extremely divergent in *C. carolina*, especially the part between the canonical helices 16 and 19 (positions 474 and 723). It was also considerably longer than in the other heteroloboseids and selected eukaryotes (*C. carolina* 250 bp, other Tetramitia 87 – 100 bp, *Pharyngomonas* 72 bp, undescribed heteroloboseid 'BB2' – 79 bp, other eukaryotes 79 – 82 bp). It was possible to reconstruct several different, often overlapping helices in the V3 region of *C. carolina* (not shown) and it was unclear which are homologous to the canonical helices 17 and 18, and which (if any) correspond to helix 17-1.

Discussion

Identity of *Creneis carolina*

The main trophic stage of *Creneis carolina* in culture is an amoeboid flagellate with a single anterior flagellum. These cells show a close connection between the flagellum and nucleus, but do not possess a feeding groove or cytostome, and they use pseudopodia as the main apparatus for locomotion and feeding. This stage superficially somewhat resembles some pelobionts such as *Mastigamoeba* (Amoebozoa: Archamoebae; see Ptáčková et al. 2013), 'hyperamoebid' slime molds such as *Didymium dachnayum* (Amoebozoa: Myxogastria; see Walker et al. 2003), and *Breviata* (Breviatea; a relative of apusomonads and opisthokonts; see Brown et al. 2013). In contrast

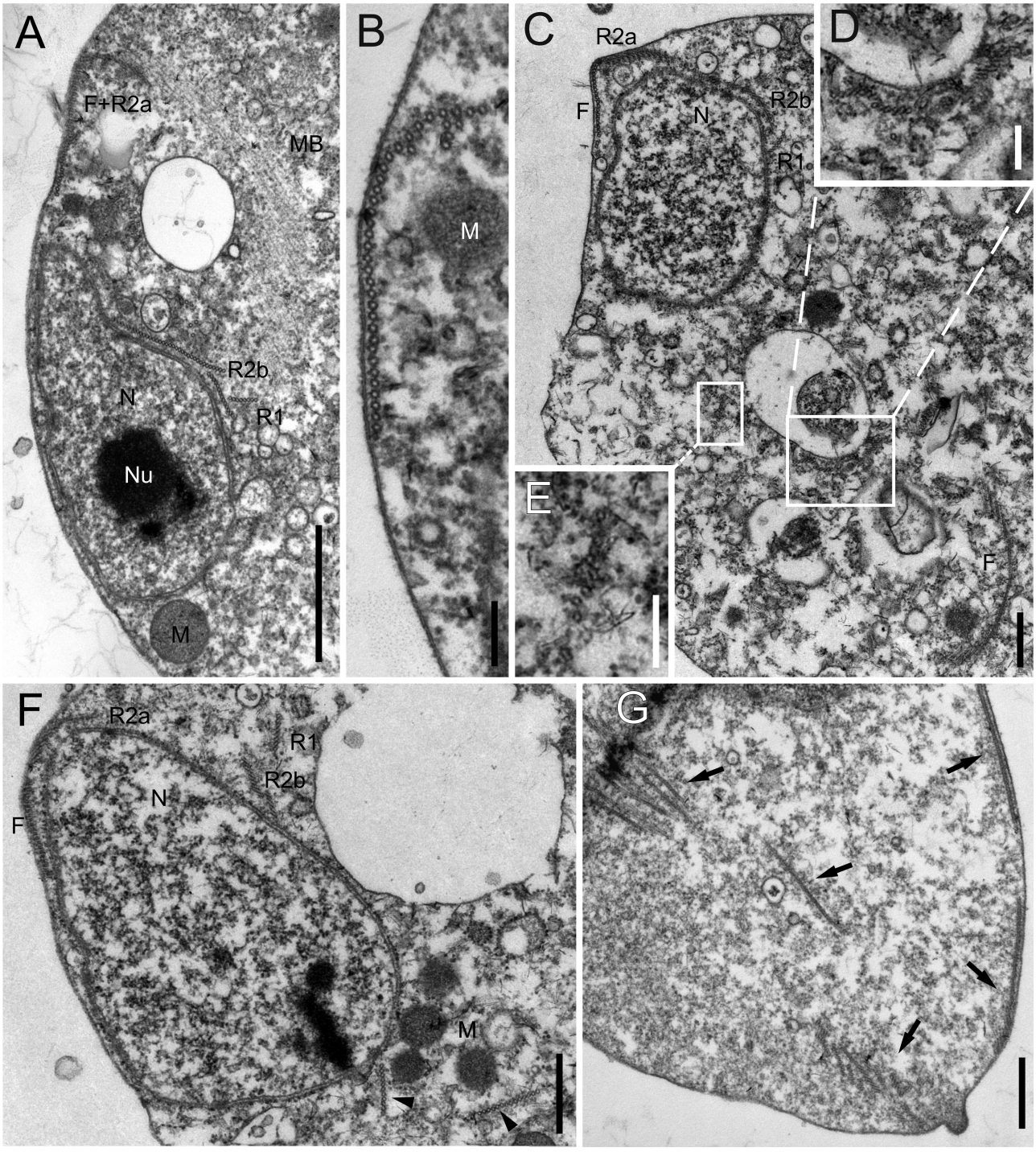

Figure 9. Transmission electron micrographs of *Creneis carolina* – more distal arrangements of microtubular ribbons. **A.** Multiple ribbons near nucleus derived from R2b and R1. Note also central nucleolus in the nucleus, and microfibrillar bundle. **B.** Transverse section through the 'thick fibre' (associated R2a and right arm of the 'F' element). **C.** Low magnification view of same cell as in Figure 8, showing left arm of the 'F' running deeply into the cell, and microtubular ribbons derived from the associated MTOC-like region. **D, E.** Detail of ribbons derived from MTOC-like region. **F.** Microtubular structures associated with nucleus, including two microtubular ribbons derived from the MTOC-like region near posterior end of nucleus (arrowheads). Note also the cluster of mitochondrion-related organelles. **G.** Section through the cell posterior showing microtubular ribbons.

to all of these amoeboid uniflagellates, *C. carolina* is able to transform into a multiflagellate cell that is not particularly similar to any eukaryote that we are aware of. The organism that most closely resembles the multiflagellate stage may be *Multicilia marina*. However typical *Multicilia* cells have little or no organization about a main axis and the flagella are relatively evenly distributed about the cell (see Mikrjukov and Mylnikov 1998), unlike *C. carolina*, where the flagella emerge only from the well-defined anterior pole and a single radial ring. The multiflagellates were transient and short-lived in the culture we studied, and their numbers decreased during several years of cultivation. A possible explanation of this change is sufficient (and relatively stable) amounts of bacterial prey in the culture. Unfortunately, we have not been able to induce the trophic form to produce multiflagellates using different culture media or subculturing intervals.

Besides these two life stages, a few amoeboid biflagellates and aflagellated non-motile forms were observed. All the forms of *C. carolina* are uninucleate and the nuclei all share a distinctive elongate, curving shape. Our data concerning most of the stages are limited and we are unable to connect them in the context of a life cycle. However, it is likely that the biflagellated uninucleate cells do not represent division stages, because their flagella are fully developed. In contrast, the flagella of the apparently dividing cells depicted in Figure 2H and 2I are rather short and possibly not fully mature. The biflagellate cells might represent a transitional stage between the trophic stage and the multiflagellate, since the latter possesses two anterior flagella besides the multiple lateral ones. It is well known that the taxon Heterolobosea includes species with multiple life history stages, typically trophic amoebae, trophic or dispersive flagellates, and cysts. Although *Creneis carolina* is able to transform into various life stages, none of them resembles those of typical heteroloboseids. In particular, heteroloboseid flagellates are not highly amoeboid and do not produce pseudopodia; they also almost invariably have 2 or 4 flagella, rarely more, and never a single flagellum (Pánek and Čepička 2012). Conversely, the amoeba stage of typical heteroloboseids has no basal bodies/centrioles nor any other recognizable part of the flagellar apparatus (Fulton 1977; Fulton and Dingle 1971; Lee 2010). Consequently, the affinity of *C. carolina* with Heterolobosea revealed by molecular phylogenetics was very surprising from the perspective of superficial morphology and life history.

Our molecular phylogenetic results convincingly showed that *Creneis carolina* is a member of Tetramitia, the most diverse group of Heterolobosea (Cavalier-Smith and Nikolaev 2008). Its precise phylogenetic position within Tetramitia remains uncertain, however. Tetramitia share the unique '17_1' helix in the secondary structure of the V3 hypervariable region of the SSU rRNA molecule (Cavalier-Smith and Nikolaev 2008; Harding et al. 2013; Nikolaev et al. 2004; Park and Simpson 2011; Park et al. 2012; Wuyts et al. 2001). Because the V3 region of *C. carolina* is extremely divergent, we were unable to identify its precise secondary structure. Nevertheless, the SSU rRNA of *C. carolina* is likely to contain a 17_1 helix since its V3 region is considerably longer than that of other heteroloboseids.

The taxon Tetramitia is currently divided into seven major clades. Clades I – VI were defined by Pánek et al. (2012) and *Selenaion koniopes* is a recently discovered additional lineage (Park et al. 2012), here labelled as clade VII (Fig. 12). Our phylogenetic analyses recover two possible phylogenetic positions of *Creneis carolina*: (1) as a sister lineage to Psalteriomonadidae, which is supported by more analyses, or (2) with or within clade IV, otherwise comprising *Percolomonas cosmopolitus* and *Stephanopogon* spp. (also known as Percolatea; Cavalier-Smith 2003; Cavalier-Smith and Nikolaev 2008), which is supported by some analyses of the SSU rRNA data set alone. As *C. carolina* represents the longest branch in the tree, it is expected that its position will be affected by artifacts like long branch attraction.

Creneis carolina resembles psalteriomonads in being an obligate anaerobe that lacks mitochondrial cristae. This does lend some support to a relationship with Psalteriomonadidae. However, Psalteriomonadidae are probably not the only heteroloboseids with the capability to live under

Abbreviations: F – 'F' element; N – nucleus; MB – microfibrillar bundle; M – mitochondrion-related organelles; R1 – putative microtubular root R1; R2 – microtubular root R2; R2a – the first part of split R2 closer to the basal body; R2b – the second part of split R2; arrows – microtubular ribbons of uncertain origin (in the posterior cell part); arrowhead – microtubular ribbons derived from MTOC-like region. Scale bars: 1 μm for A; 200 nm for B, D, E; 500 for C, F, G.

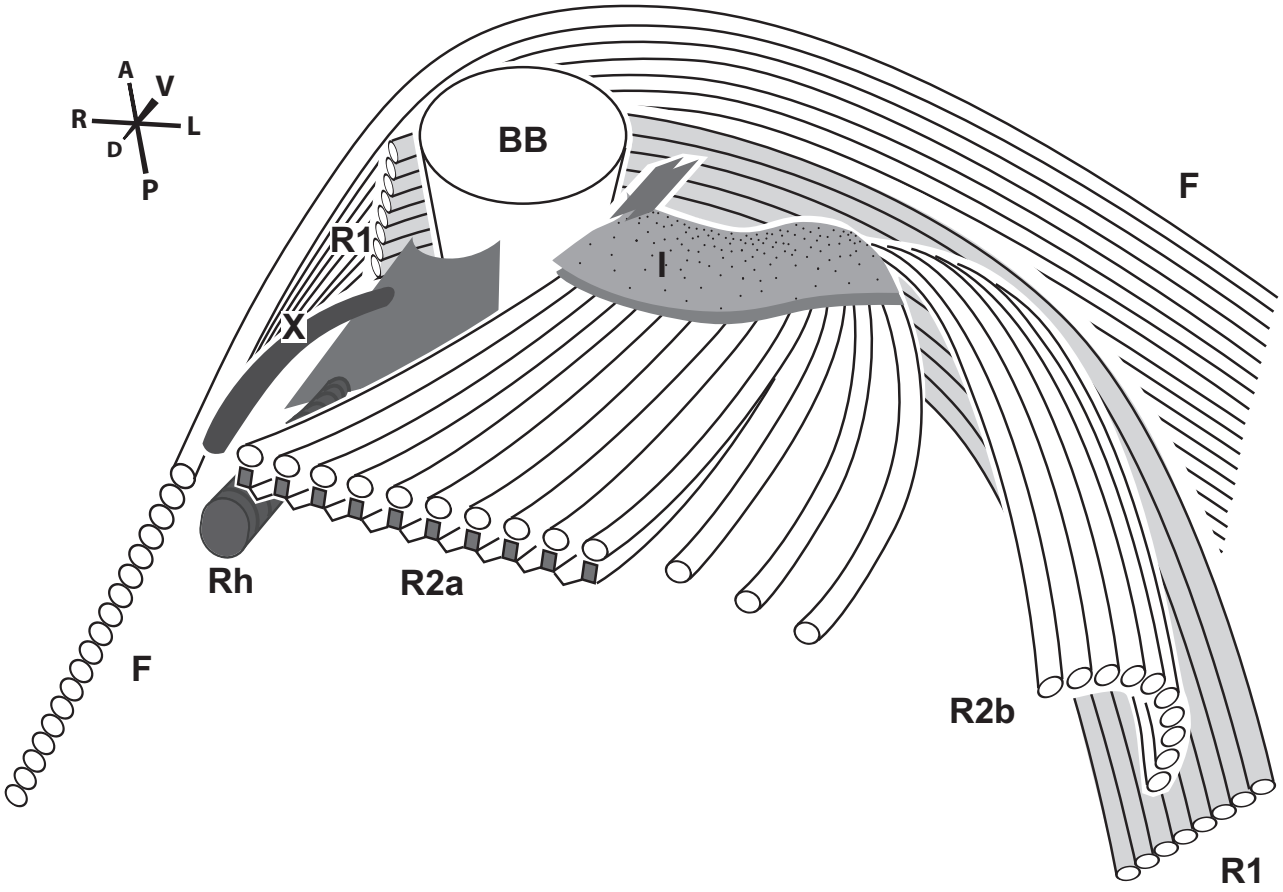

Figure 10. Diagram showing the flagellar apparatus at the anterior end of the amoeboid uniflagellate cells of *Creneis carolina*. The apparatus is shown looking at the ventral face of the cell, somewhat from the posterior end and right side. ‚BB' represents the basal body (transition zone and axoneme proper not shown). ‚R1', ‚R2a', ‚R2b' and ‚F' represent the major microtubular elements (see text); R1 is shaded for contrast only. ‚Rh' represents the Rhizoplast, ‚I' the ‚I fibre' and ‚X' the ‚X element' (see text). Nucleus and individiual microtubules (IMts) are not shown.

anaerobic or microoxic conditions. For example, *Pleurostomum flabellatum*, which is unrelated to the Psalteriomonadidae, lives in extremely hypersaline waters with low oxygen solubility and might lack mitochondrial cristae as well (Park et al. 2007). In addition, the genome of *Naegleria gruberi* encodes enzymes central to hydrogenosome-like anaerobic metabolism (Fritz-Laylin et al. 2010).

Possible similarities between *Creneis carolina* and Percolatea are not as evident, because *Stephanopogon* also has a very divergent morphology relative to other Heterolobosea. There is, however, electron-dense material associated with the origins of the axonemal central pair in *Creneis* that shows similarity to an electron-dense globule in *Stephanopogon* (Patterson and Brugerolle 1988; Yubuki and Leander 2008), although it is questionable whether this structure is also present in *Percolomonas* (Lee et al., in press; see below).

Despite all efforts we were not able to elucidate the precise position of *C. carolina* within Tetramitia. Nonetheless, its discovery significantly extends the known diversity of morphology and life history within Heterolobosea. Because *C. carolina* does not closely resemble other known heteroloboseids, we have decided to accomodate it in a new family, Creneidae fam. nov.

Flagellar Apparatus of *Creneis carolina* and Comparison with Other Eukaryotes

The flagellar apparatus (or mastigont) of eukaryotes is a complex structure composed of the basal bodies, their axonemes and the associated

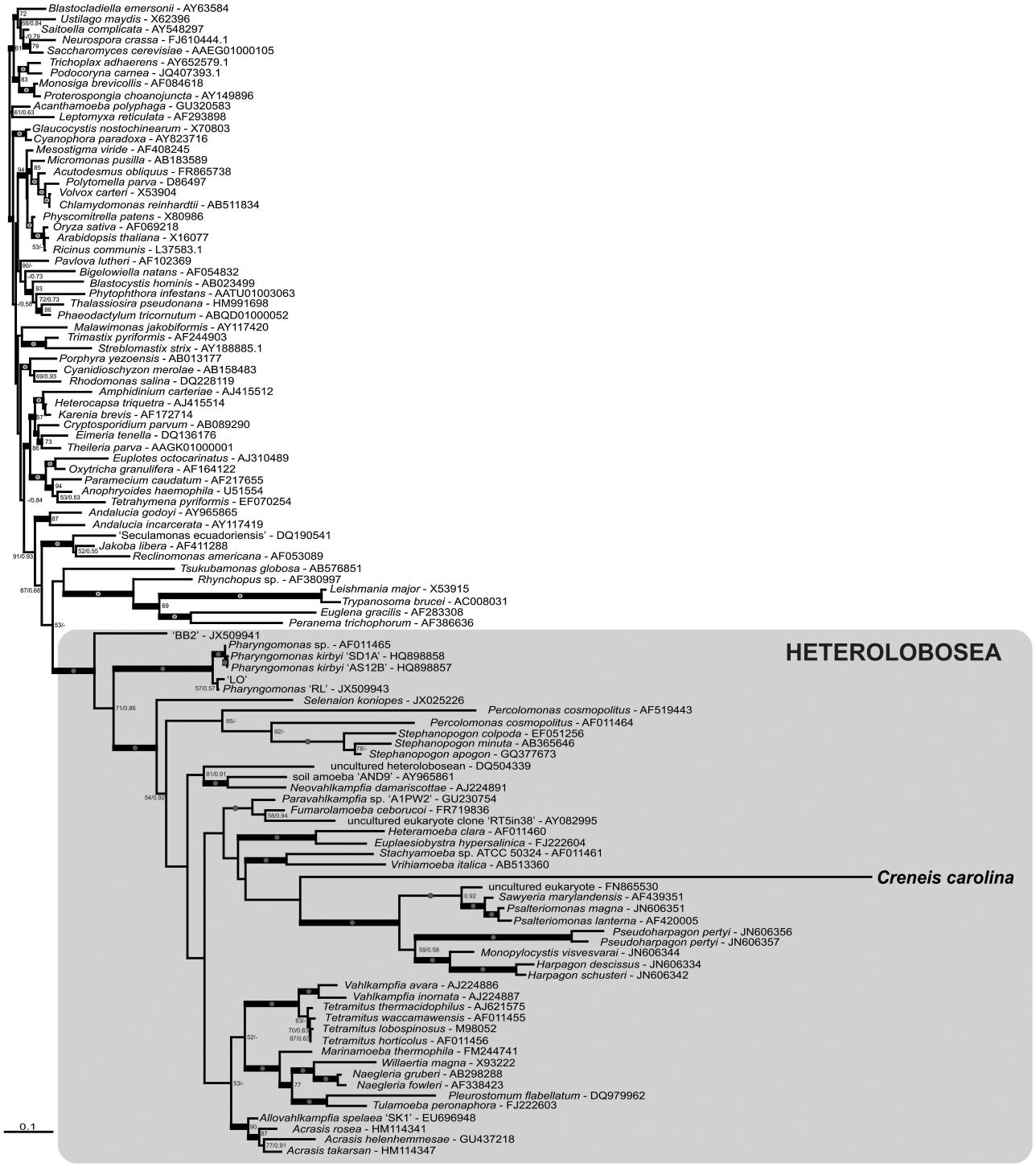

Figure 11. Phylogenetic tree of eukaryotes based on sequences of the gene for small subunit ribosomal rRNA. The tree is based on 1270 well-aligned positions from 105 taxa. The topology was inferred in RAxML using maximum likelihood with the GTRGAMMAF model of sequence evolution. The values at nodes represent RAxML bootstrap percentages followed by PhyloBayes posterior probabilities. RAxML bootstrap values > 95% are marked by grey circles, PhyloBayes posterior probabilities > 0.95 are marked by thick branches. Values lower than 50% or 0.5 are not marked or marked by ,,-".

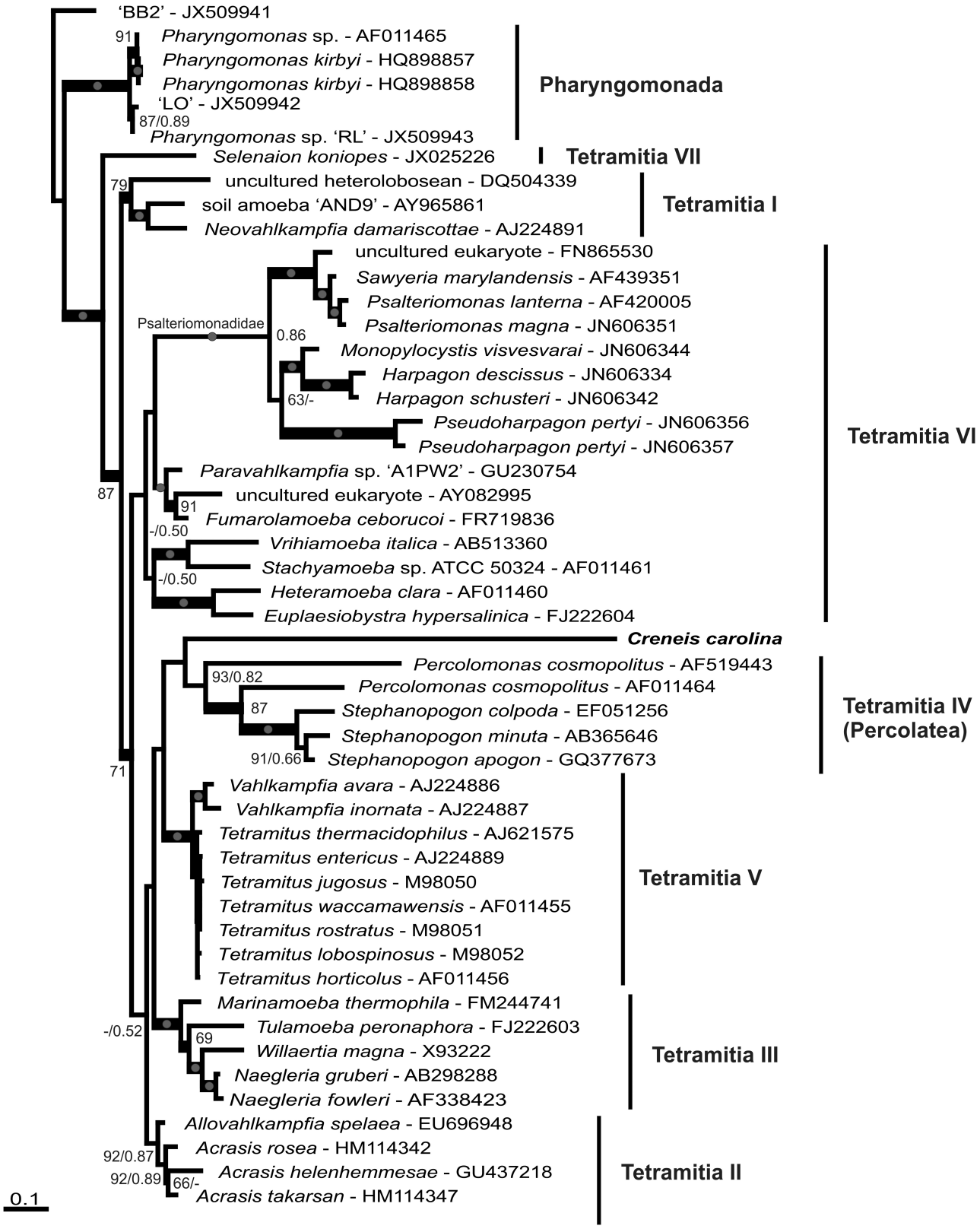
'BB2' - JX509941
91
Pharyngomonas sp. - AF011465
Pharyngomonas kirbyi - HQ898857
Pharyngomonas kirbyi - HQ898858
'LO' - JX509942
87/0.89
Pharyngomonas sp. 'RL' - JX509943
Pharyngomonada
Selenaion koniopes - JX025226
Tetramitia VII
79
uncultured heterolobosean - DQ504339
soil amoeba 'AND9' - AY965861
Neovahlkampfia damariscottae - AJ224891
Tetramitia I
uncultured eukaryote - FN865530
Sawyeria marylandensis - AF439351
Psalteriomonas lanterna - AF420005
Psalteriomonadidae
0.86
Psalteriomonas magna - JN606351
Monopylocystis visvesvarai - JN606344
Harpagon descissus - JN606334
63/-
Harpagon schusteri - JN606342
Pseudoharpagon pertyi - JN606356
Pseudoharpagon pertyi - JN606357
Tetramitia VI
Paravahlkampfia sp. 'A1PW2' - GU230754
87
uncultured eukaryote - AY082995
91
Fumarolamoeba ceborucoi - FR719836
-/0.50
Vrihiamoeba italica - AB513360
Stachyamoeba sp. ATCC 50324 - AF011461
-/0.50
Heteramoeba clara - AF011460
Euplaesiobystra hypersalinica - FJ222604
Creneis carolina
Percolomonas cosmopolitus - AF519443
93/0.82
Percolomonas cosmopolitus - AF011464
87
Stephanopogon colpoda - EF051256
Stephanopogon minuta - AB365646
91/0.66
Stephanopogon apogon - GQ377673
Tetramitia IV (Percolatea)
71
Vahlkampfia avara - AJ224886
Vahlkampfia inornata - AJ224887
Tetramitus thermacidophilus - AJ621575
Tetramitus entericus - AJ224889
Tetramitus jugosus - M98050
Tetramitus waccamawensis - AF011455
Tetramitus rostratus - M98051
Tetramitus lobospinosus - M98052
Tetramitus horticolus - AF011456
Tetramitia V
Marinamoeba thermophila - FM244741
-/0.52
Tulamoeba peronaphora - FJ222603
69
Willaertia magna - X93222
Naegleria gruberi - AB298288
Naegleria fowleri - AF338423
Tetramitia III
Allovahlkampfia spelaea - EU696948
92/0.87
Acrasis rosea - HM114342
92/0.89
66/-
Acrasis helenhemmesae - GU437218
Acrasis takarsan - HM114347
Tetramitia II
0.1

Figure 12.

cytoskeleton. It usually serves as an organizing centre for much of the microtubular cytoskeleton and has relatively conservative architecture within particular eukaryotic lineages (for details see Moestrup 2000; Yubuki and Leander 2013).

Heterolobosea, including *Creneis carolina*, belongs to the supergroup Excavata. Many excavates have a complex flagellar apparatus composed of a relatively large number of microtubular and non-microtubular components. Typical excavates possess two or four flagella, of which the posterior flagellum is the eldest. The basal body of the posterior flagellum is associated with three distinct microtubular structures (R1, split R2, singlet root) and several non-microtubular fibers (A, B, C, I). In brief, R1 is attached to the C fibre (a form of multilayered structure or MLS; see Yubuki and Leander 2013) and supports the left side of a ventral feeding groove. The curved R2 is initially supported on its concave (cell-membrane-side) face by the I fibre, and splits such that the outer part (furthest from the basal body) supports the right side of the feeding groove together with the B fibre, while the inner part supports either the left, centre or right floor of the groove, depending on the taxon. The basal body of the second oldest flagellum usually anchors microtubular root R3, which typically originates a 'dorsal fan' of microtubules that supports the dorsal cell membrane (Simpson 2003; Yubuki et al. 2013).

Heteroloboseid flagellates can retain a modified and generally reduced version of the excavate flagellar apparatus. In flagellates with a feeding groove (e.g. many Psalteriomonads), the splitting R2 and associated I fibre form the main support for the groove, with the outer portion of R2 supporting the right wall, while the inner portion supports the left wall (Broers et al. 1990, 1993; Brugerolle and Simpson 2004; note that R2 was erroneously labeled R1 in Brugerolle and Simpson 2004, and Park et al. 2007). The outer R2 is not supported on its cell-membrane side by a B fibre, instead the convex (more cytoplasmic) face of R2 is supported by a striated rhizoplast that takes the place of the A fibre. The R2, I fibre and rhizoplast system is typically retained even if the feeding structure is substantially modified or lost entirely – e.g. *Tetramitus*, *Naegleria* and *Pleurostomum* (Balamuth et al. 1983; Brugerolle and Simpson 2004; Park et al. 2007). The R1/C fibre system no longer supports the left wall of the groove, and is usually absent (or possibly highly reduced). However, the deep branching heteroloboseid *Pharyngomonas kirbyi* retains a conspicuous R1 and C fibre, and also has a small spur-like structure that might represent a reduced B fibre (Park and Simpson 2011). A distinct R3 appears to be absent in Heterolobosea, and the dorsal microtubules often make a sweeping arc around the anterior/dorsal side of the flagellar apparatus, for example in psalteriomonads and *Tetramitus* (Broers et al. 1990, 1993; Brugerolle and Simpson 2004; Outka and Kluss 1967).

In *Creneis carolina*, the 'F' element takes a sweeping path around the dorsal side of the flagellar apparatus and appears to be the homolog of the dorsal microtubules of typical Heterolobosea, albeit organised into an unusually robust ribbon of adjacent microtubules. The element we have named as R2 is similar to the typical R2 of Heterolobosea in several respects: it is supported on the convex side near its origin by a latticework element that resembles an I fibre, while the other side of R2 (the cytoplasmic side) is associated with a striated element identified as a probable rhizoplast. Furthermore, it splits into two halves, with the portion closest to the basal body (the 'inner' part; 'R2a') remaining associated with rhizoplast. The interconnected doubled-appearance supporting spokes that develop on the cytoplasmic side of each R2a microtubule (not to be confused with the possible I fibre homologue on the cell-membrane side – see above), appear to be present also on the leftmost microtubules of the outer portion of R2 (oR2) in at least some psalteriomonads (fig. 10a, b in Pánek et al., in press; see also fig. 2d in Brugerolle and Simpson 2004), consistent with homology of the underlying microtubular roots. Similar spokes might also be present on a band of microtubules that support the gullet of *Tetramitus* (fig. 18 in Balamuth et al. 1983); though it is unclear where this band originates in *Tetramitus*. More uncertainly, the R2 in *C. carolina* is associated with a spur-like element

Figure 12. Phylogenetic tree of Heterolobosea based on sequences of gene for small subunit ribosomal rRNA. The tree is based on 1235 well-aligned positions from 50 taxa. The topology was inferred in RAxML using maximum likelihood with the GTRGAMMAF model of sequence evolution and rooted on the sequence of 'BB2', following Figure 11. The values at nodes represent RAxML bootstrap percentages followed by PhyloBayes posterior probabilities. RAxML bootstrap values > 95% are marked by grey circles, PhyloBayes posterior probabilities > 0.95 are marked by thick branches. Values lower than 50% or 0.5 are not marked or marked by ‚-'.

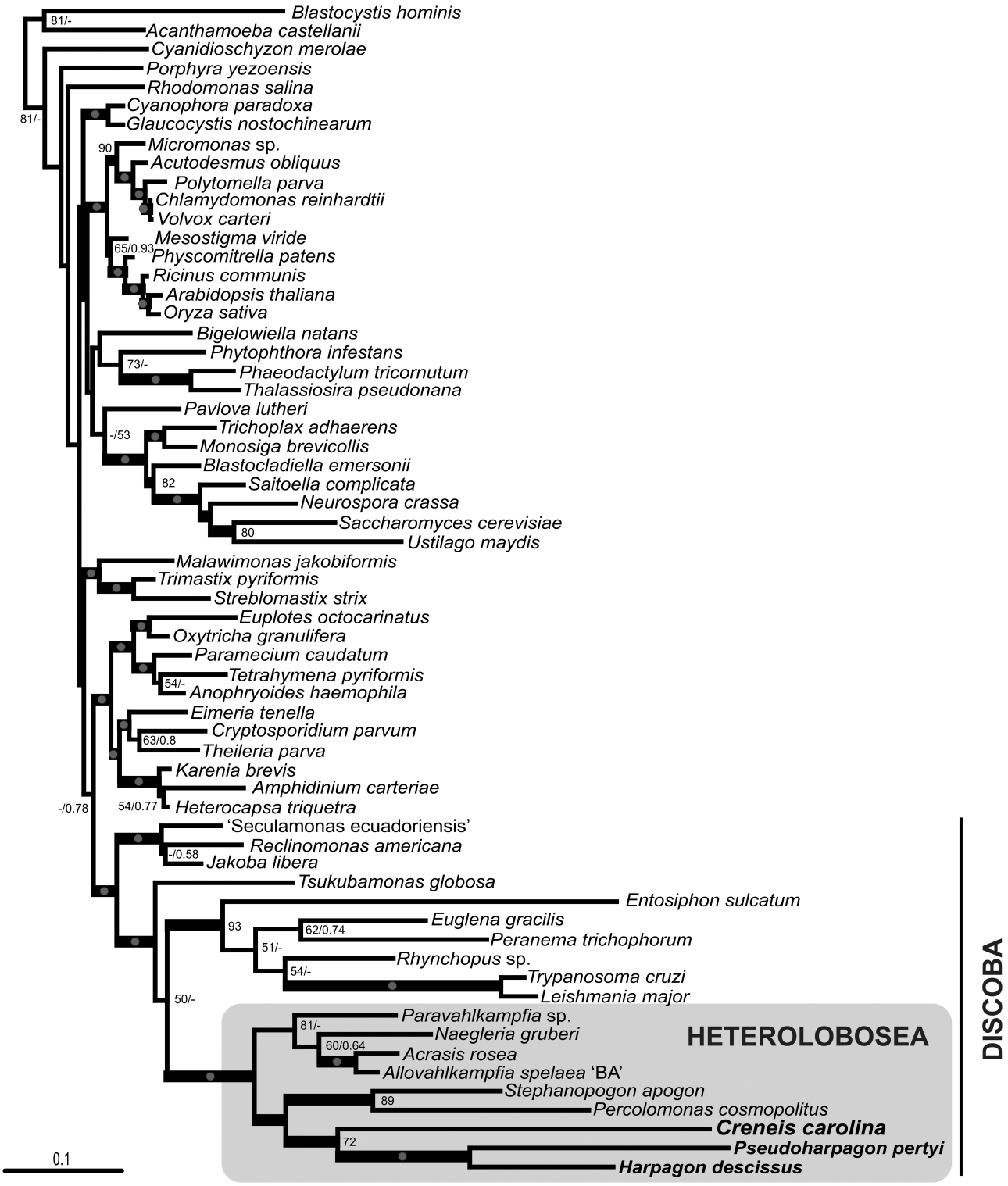

Figure 13. Phylogenetic tree of eukaryotes based on a concatenation of the gene for small subunit ribosomal rRNA and inferred amino acid sequences for α- and β-tubulin. The tree is based on 1985 well-aligned positions total from 62 taxa. The topology was inferred in RAxML using maximum likelihood and a partitioned model of sequence evolution (GTRGAMMAF for nucleotides; PROTGAMMALGF for the amino acid partitions). The values at nodes represent RAxML bootstrap percentages followed by PhyloBayes posterior probabilities.

similar to the possible B fibre homologue identified in *Pharyngomonas* (Park and Simpson 2011). However, in *C. carolina* the R2 unit originates more to the left side of the basal body, not the right side, and the rhizoplast originates to the right of R2, not to the left as in comparable heteroloboseids (Brugerolle and Simpson 2004; Park et al. 2007; Park and Simpson 2011). Further, it is the inner portion of the R2 ('R2a') that runs down the right side of the cell, whereas it is the outer portion of R2 that supports the right wall of the groove in psalteriomonads (RF in Broers et al. 1993). Conversely, in *Pleurostomum* the branch of the R2 that remains associated with the rhizoplast (and is probably also the 'inner' part) supports the equivalent of the left side of the cell (Park et al. 2007), not the right as in *Creneis*.

In short, the R2 of *Creneis* largely resembles the R2 of other Heterolobosea, except that is in mirror image in our reconstruction. If this structure is truly homologous to R2 then it, or the entire flagellar apparatus system, may have undergone a reversal of chirality relative to the flagellum. Alternately, it is possible that the chirality of the flagellum and basal body has become inverted, and that our reconstruction of the entire cell, which is based on the absolute orientation of the flagellum (Sleigh 1988) is inverted. As far as we are aware these would be highly unusual or unprecedented evolutionary developmental changes. The molecular process that controls the development of the flagellar apparatus is poorly known even in model systems like *Chlamydomonas* (see Marshall 2012). Nevertheless, amoebae of heteroloboseids (which lack the flagellar apparatus entirely) transform into flagellates with the same flagellar apparatus chirality as pure flagellates (e.g. Brugerolle and Simpson 2004), and there a strong case has been made that the general orientation and function of the R2 microtubular root has been conserved across the tree of extant eukaryotes after descent from the last eukaryotic common ancestor (Cavalier-Smith 2013; Heiss et al. 2013; Yubuki and Leander 2013). This illustrates that the general architecture of the flagellar apparatus is robust and conservative, making the change that may have occurred in *Creneis* all the more surprising.

The other major microtubular element closely associated with the basal body is 'R1', which originates on the dorsal/right side of the basal body. It then heads left, which is the direction taken by the R1 root of *Pharyngomonas* and of typical excavates (Park and Simpson 2011; Yubuki et al. 2013), albeit the R1 in these other taxa runs immediately under the cell membrane. Identifying this root as a true R1 root is not straightforward: if either of the reversals of chirality suggested above had occurred then R1 in *Creneis* would be expected to travel (what appears to be) rightwards. Another reason to doubt that this element is a true R1 is that an elongated R1 (i.e. extending beyond the immediate vicinity of its attached basal body) has not been documented previously in Tetramitia, although it is possible that *Creneis* may be a relatively deep-branching lineage of Tetramitia, in which case retention of an R1 could be parsimonious. Finally, if the identification of R2 (see above) is mistaken, this root would then be a candidate for being the true homolog R1 of *Creneis*, based on its position relative to the basal body. We think this is unlikely because there are no other special similarities with the R1 roots of other taxa (for example the double-leaved material discussed above does not resemble a C-fibre or MLS), and, as already mentioned, because R2 is always more extensively developed than R1 in Tetramitia (see Brugerolle and Simpson 2004, noting that the labels 'R1' and 'R2' are reversed in that publication).

A few other features of the cell ultrastructure are noteworthy. The absence of identifiable Golgi bodies is characteristic of Heterolobosea (Page and Blanton 1985), though not diagnostic. The lack of ER enveloping of the mitochondria is atypical for Heterolobosea (Page and Blanton 1985), but has been observed in several unrelated heteroloboseid lineages including, interestingly, members of both Percolatea and Psalteriomonadidae (Fenchel and Patterson 1986; Pánek et al. 2012; Park et al. 2007; Park and Simpson 2011; Patterson and Brugerolle 1988;Yubuki and Leander 2008). The presence of only a single basal body in the kinetid is highly atypical. Almost all other heteroloboseids have two or four basal bodies per kinetid (Park and Simpson 2011; Patterson et al. 2000), with the single documented instance of monokinetids being the special case of the multiflagellate taxon *Stephanopogon* (see Patterson and Brugerolle 1988; Yubuki and Leander 2008).

Yubuki and Leander (2008) identified two possible ultrastructural synapomorphies of Percolatea: (1) the central pair of axonemal microtubules terminates with a ball-like, electron dense globule near the transitional plate, and (2) the presence of dense

RAxML bootstrap values > 95% are marked by grey circles, PhyloBayes posterior probabilities > 0.95 are marked by thick branches. Values lower than 50% or 0.5 are not marked or marked by ,,-".

Table 1. Comparison of the ultrastructural features of *Creneis carolina* gen. et sp. nov. (amoeboid uniflagellate stage) with flagellates of Discoba. Table summarizes presence of elements that are typically associated with the oldest flagellum and two other important characters (the dorsal fan of microtubules and mitochondrial cristae).

	Ventral groove	# BBs/flagella	R1	split R2	SR	A fibre (rhizoplast)	B fibre	I fibre	C fibre (MLS)	F	MC
***Creneis carolina* gen. et sp. nov.**	-	1/1	?	+[a]	-	Rh[b]	?[c]	+	-	+	NC
Heterolobosea (Tetramitia)	±	variable; >2/>2	-	+	-	Rh[b]	-	+	-	+	D(NC)
Heterolobosea (*Pharyngomonas*)	+	4/4	+	+	-	?	?[c]	+	+	+	D
Euglenozoa (ancestral state)	-	2/2	+	-	-	-	-	-	-	+	D
Tsukubamonas globosa	+	4/2	-	+	+	A	+	+	-	-	T
Jakobida	+	2/2	+	+	+	A	+	+	+	+	T(F/NC)

+: present; -: absent; ±: present only in some species; ?: identification is not straightforward; # BBs: number of basal bodies per cell; F: dorsal fan of microtubules; MC: mitochondrial cristae (D: discoidal; F: flattened; NC: no cristae; T: tubular; parentheses: less common or derived type); Rh: rhizoplast; SR: singlet root. Data from other members of Discoba were adopted from Park and Simpson (2011) and Yabuki et al. (2011).
[a]Microtubular root R2 of *Creneis* largely resembles the R2 of other Heterolobosea, except that it is mirror image of canonical R2.
[b]Rhizoplast of Heterolobosea is positionally similar to A fibre, thus, these two structures might be homologous. On the other hand, A fibre is less distinct and non-striated. The putative rhizoplast of *Pharyngomonas* is amorphous and broad structure. The rhizoplast of *C. carolina* originates to the right of R2, not to the left as in other studied heteroloboseids.
[c]Spur-like element of *Creneis carolina* is similar to the possible B fibre homologue identified in *Pharyngomonas* by Park and Simpson (2011).

fibrous sheets attached to the proximal ends of basal bodies. Lee et al. (in press) noted that the first is unlikely to be present in *Percolomonas*, since it is not seen in published longitudinal sections of the transitional zone (see Fenchel and Patterson 1986), and the second may not be particular to Percolatea, since it is documented in at least one *Percolomonas*-like species (Bernard et al. 2000) that was recently shown to actually be a psalteriomonad (Pánek et al. 2012). Interestingly *Creneis carolina* arguably possesses both of these features. The dense material associated with the central pair origin does resemble that of *Stephanopogon* in its location, although it is less globular in appearence. The fibrous sheet that is present below the basal body is less dense than the structures in *Percolomonas*, *Stephanopogon*, and (at least some) psalteriomonads (Bernard et al. 2000; Fenchel and Patterson 1986; Patterson and Brugerolle 1988; Yubuki and Leander 2008), and the striated connection that attaches the sheet to the basal body in *Creneis* was not documented in the other taxa. More complete data from other heteroloboseids would be valuable in clarifying the phylogenetic significance of these feature.

In summary, the morphology of *Creneis carolina*, especially the architecture of the flagellar apparatus, is unique not only among Heterolobosea, but Excavata in general. The mastigont contains elements that are probably homologous to certain structures typical for the two groups, but its organization is highly unusual (Table 1). Moreover, the flagellar apparatus of *C. carolina* may have undergone striking evolutionary developmental changes not seen in other known eukaryotes.

Taxonomic Summary

Eukaryota: Excavata: Discoba: Disciscristata: Heterolobosea: Tetramitia

Creneidae fam. nov.

urn:lsid:zoobank.org:act:8F9B5D49-29C2-4A21-883D-9C35AAD26382

Diagnosis: Obligately anaerobic or microaerophilic Heterolobosea. Mitochondria without cristae. Life cycle includes an amoeboid flagellate with a single, long anterior flagellum and a flagellate with more than ten flagella. Ventral groove and cytostome absent.

Type genus: *Creneis* gen. nov.

Etymology: Crene- (considered as the stem of the name) + -idae

Creneis gen. nov.

urn:lsid:zoobank.org:act:87C85F4B-67D1-47E7-AF07-D97E452EA1FF

Diagnosis: as for family Creneidae.

Type species: *Creneis carolina* sp. nov. by monotypy.

Etymology: Named after Creneis, one of sea nymphs (Nereids) in Greek mythology. Feminine gender.

Creneis carolina sp. nov.

urn:lsid:zoobank.org:act:2E38C34D-2510-48DA-B672-5CCA2E112E66

Diagnosis: Marine *Creneis* with amoeboid flagellates ca. 20 μm long and ca. 13 μm wide; moving over the substrate through a broad, hyaloplasmic, and extremely flattened anterior non-eruptive front. The posterior end usually with a mass of adhesive filaments up to 40 μm long. Apically located nucleus is elongated, ca. 5.5 μm long and ca. 2.5 μm wide, usually curved. Single flagellum ca. 43 μm long. Multiflagellate cells elongate or rounded, fast-swimming, with approximately 14 flagella, 20 – 25 μm long and 8 – 9 μm wide.

Type locality: Peggy's Cove, Nova Scotia, Canada. 44 °29'N, 63 °55'W.

Habitat: Shallow anoxic coastal sediment.

Type culture: Monoeukaryotic culture PC4AM with *Creneis carolina* and unidentified bacteria, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Hapantotype: Protargol preparations of the strain PC4AM, deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic, catalogue numbers 6/20 – 6/23. Figs. 2, 3E – L, 4E – I are images from the hapantotype.

Etymology: *carolina* [Latin] – "of Charles". Named in honor of the Holy Roman Emperor Charles IV (1316 – 1378), founder of Charles University in Prague in 1348.

Methods

Organisms: The strain PC4, containing various marine protists, was obtained from anoxic marine coastal sediments in Peggy's Cove (Nova Scotia, Canada; 44°29'N, 63°55'W) in 2007, transferred into seawater 802 medium (ATCC medium 1525). This medium was also used for subsequent cultivation. The strain PC4 was maintained as a xenic culture at room temperature in 15-ml tubes with 10 ml of media and was transferred into fresh medium once a week. Later on, two monoeukaryotic cultures, strain PC4AM of *Creneis carolina* sp. nov., gen. nov., and strain PC4BIC of *Monopylocystis visvesvarai* (Pánek et al. 2012) were derived from the original isolate PC4. The strain PC4AM of *C. carolina* was cultured as per the original strain PC4.

Light and fluorescence microscopy: The morphology of living, protargol-stained and DAPI- stained cells of *Creneis carolina* was examined under using an Olympus BX51TF light microscope equipped with a DP70 color camera (Olympus) using a 100x oil-immersion lens. Living cells were observed using Differential Interference Contrast (DIC). Protargol-stained preparations were observed by bright-field microscopy and prepared as follows: moist films spread on cover slips were prepared from pelleted cultures obtained by centrifugation at 500 *g* for 8 minutes. The films were fixed in Bouin-Hollande's fluid for 10 hours, washed with 70% ethanol, and stained with 1% protargol (Bayer, I. G. Farbenindustrie) following Nie's (1950) protocol. To visualize the nucleus, the cells of *C. carolina* were stained by DAPI (4',6-diamidino-2-phenylindole) following the protocol of Dawson et al. (2008), as modified by Zubáčová et al. (2013).

Transmission electron microscopy: Cells of *Creneis carolina* were pelleted by centrifugation at 500 *g* for 8 minutes, resuspended in a solution containing 2.5% glutaraldehyde (Polysciences) and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.2), and fixed at room temperature for 4 hours. After three washes in 0.1 M cacodylate buffer (15 minutes each), the cells were postfixed with 2% OsO_4 in 0.1 M cacodylate buffer for 3 hours. After washing three times as above the fixed cells were dehydrated in an acetone series and embedded in Epon resin (Poly/Bed 812/Araldite, Polysciences). Ultrathin sections were cut using a diamond knife on an Ultracut E ultramicrotome (Reichert), stained with lead citrate and saturated uranyl acetate, and examined using a TEM JEOL 1011 transmission electron microscope.

DNA isolation, amplification, cloning and sequencing: Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The almost complete SSU rDNA of *Creneis carolina* was amplified using primers Euk A (5'-CTGGTTGATCCTGCCAG-3') and Euk B (5'-TGATCCTTCTGCAGGTTCACCTAC-3') (Medlin et al. 1988) with an annealing temperature of 50 °C. The α-tubulin genes of *Creneis carolina* strain PC4AM, *Harpagon descissus* strain TEXEL, and *Pseudoharpagon pertyi* strain EVROS2N were amplified using nested PCR. The primary PCR was conducted using primers AtubA (5'-RGTNGGNAAYGCNTGYTGGGA-3') and AtubB (5'-CCATNCCYTCNCCNACNTACCA-3') (Edgcomb et al. 2001), with an annealing temperature of 50 °C. The secondary PCR was conducted using primers 5'-TTGTACTGCYTNGARCAYG-3' and 5'-ACGTACCAGTGNACRAANGC-3' designed by Yoon et al. (2008), with an annealing temperature of 50 °C. The β-tubulin gene of *Creneis carolina* was amplified using primers BtubA (5'-GCAGGNCARTGYGGNAAYCA-3') and BtubB (5'-AGTRAAYTCCATYTCRTCCAT-3') (Edgcomb et al. 2001) with an annealing temperature of 57 °C. The PCR products were purified using the Zymoclean™ GEL DNA Recovery Kit (Zymo Research) or QIAquick PCR Purification Kit (Qiagen) and were cloned into the pGEM®-T EASY vector using the pGEM®-T EASY VECTOR SYSTEM I (Promega) and at least three clones were sequenced. The SSU rRNA gene was also sequenced directly from PCR product. Sequence data reported in this paper are available in GenBank under accession numbers KJ210081 – KJ210098.

Phylogenetic analyses: Three data sets were used to infer phylogenetic position of *Creneis carolina*. 1) the gene for SSU rRNA and 105 eukaryote taxa, 2) SSU rRNA genes from 50 heterolobosеid taxa and 3) A concatenation of the gene for SSU rRNA and inferred amino acid sequences of α- and

β-tubulin from 62 eukaryote taxa. The sequences were aligned in MAFFT (Katoh et al. 2005) using G-INS-i algorithm and the alignments were manually edited and masked in BioEdit 7.0.9.0 (Hall 1999). Maximum likelihood phylogenetic trees were estimated in RAxML version 7.2.3 (Stamatakis 2006) using the GTRGAMMAF model of sequence evolution for the SSU rRNA data sets and a three-partition model for the concatenated data set. The partitioned model consisted of the GTRGAMMAF model for the SSU rRNA nucleotides and the PROTGAMMALGF model for both amino acid partitions. Model parameters were optimized independently for each partition. Bootstrap support was estimated from 500 permutations. PhyloBayes version 2.3 (Lartillot and Philippe 2004) was run on all data sets using the CAT GTR model for the analyses of SSU rRNA and the CAT POI model for concatenated data set. The two independent runs were automatically stopped after the largest discrepancy (maxdiff) dropped below 0.3 and consensus trees and posterior probabilities were calculated. All data sets and trees generated in this study have been deposited in TreeBASE under the study accession number 15227 (http://purl.org/phylo/treebase/phylows/study/TB2:S15227).

Prediction of the secondary structure of the SSU rRNA molecule: A separate data set was created that contained the 50 SSU rRNA sequences from Heterolobosea included in data set 2 (see above) plus 11 sequences of various eukaryotes for which the secondary structure of the SSU rRNA molecule was available in the European Ribosomal RNA Database (Van de Peer et al. 2000): *Coprinus cinereus*, *Gymnodinium breve*, *Chlamydomonas reinhardtii*, *Bacillaria paxillifer*, *Zea mays*, *Palmaria palmata*, *Saccharomyces cerevisiae*, *Tetrahymena canadensis*, *Calicophoron calicophorum*, *Daphnia pulex*, and *Lonicera foveata*. The sequences were aligned in MAFFT as described above. The conservative elements of the secondary structure of the SSU rRNA molecule were identified manually by inspecting the alignment. Variable elements of *C. carolina* were modeled with mfold using the mfold Web Server http://mfold.rna.albany.edu/?q=mfold (Zucker 2003) under default parameters.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2014.05.005.

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5.4. Pánek *et al.* (dokončený manuskript)

Pánek, T., Táborský, P., Pachiadaki, M.P., Hroudová, M., Vlček, Č., Edgcomb, V.P., Čepička I. Combined culture-based and culture-independent approaches provide insights into diversity of jakobids, extremely plesiomorphic eukaryotic lineage. Rukopis je připraven k zaslání do tisku.

Součástí uvedeného manuskriptu je i osm příloh. Z nich jsou zde uvedeny přílohy S1, S2, S4, S6 a S7, které jsou důležité pro pochopení prezentované práce. Ostatní tři přílohy obsahují nepublikovaná sekvenční data, jsou proto k dispozici pouze na vyžádání u autora práce.

Combined culture-based and culture-independent approaches provide insights into diversity of jakobids, extremely plesiomorphic eukaryotic lineage

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ABSTRACT

We use culture-based and culture-independent approaches to discover diversity and ecology of anaerobic jakobids (Excavata: Jakobida), an overlooked, deep-branching lineage of free-living nanoflagellates related to Euglenozoa. It belongs among a few lineages of nanoflagellates frequently detected in anoxic habitats by PCR-based studies, however only two strains of a single jakobid species have been isolated from those habitats. We recovered 712 environmental sequences and cultured 21 new isolates of anaerobic jakobids that represent at least 10 different species. Two species have never been detected by environmental, PCR-based methods, and at least 4 still remain uncultured. Our phylogenetic analyses based on SSU rDNA and six protein-coding genes showed that anaerobic jakobids constitute a clade of morphologically similar, but genetically and ecologically diverse protists – Stygiellidae fam. nov. At least one subclade of exclusively environmental sequences contains extremophiles. Our investigation combines culture-based and environmental molecular-based approaches to capture a wider extent of species diversity and shows Stygiellidae as a group that ordinarily inhabits anoxic, sulfide- and ammonium-rich marine habitats worldwide.

Keywords: cryptic species / environmental clones / marine communities / species diversity

INTRODUCTION

Extreme environments host inhabitants from of all three domains of life. Nonetheless, the terms ‘extremophile’ and ‘extremotroph’ still conjure up images of prokaryotes. However, increasing numbers of environmental studies show that heterotrophic protists, algae, and Fungi are also able to flourish in physical and geochemical extremes (Rothschild and Mancinelli, 2001). Prokaryotic biomass occurring in such environments provides a food source for bacteriovores tolerating these conditions. Thus, certain lineages of heterotrophic protists thrive in hot springs, hypersaline lakes, hydrothermal vents, anoxic sediments, DHABs, and similar habitats that are often considered to be unfavorable for eukaryotes (e.g., Atkins *et al.*, 2002; Edgcomb and Orsi, 2013).

Decreasing sequencing costs have contributed to the generation of huge amounts of new genetic information from environmental samples that are providing novel insight into eukaryotic diversity and revealing diverse communities of protists in virtually all types of habitats including ‘extreme’ ones (e.g., Alexander *et al.*, 2009; Edgcomb *et al.*, 2011a, b; Amaral Zettler *et al*., 2002).

The most commonly used marker in such PCR-based environmental studies is the small subunit ribosomal RNA gene (SSU rDNA) or its hypervariable regions V4 and V9 (Guillou *et al*., 2013; Lie *et al*., 2014; Pawlowski *et al*., 2012). Using this marker, environmental studies indicated that the real species diversity of protists may be orders of magnitude greater than previously thought (Pawlowski *et al*., 2012). On the other hand, observed genetic diversity is not easily transferable into the number of species because divergence in a single molecular marker may not correspond with an equivalent level of phenotypic differentiation among organisms (Logares *et al.*, 2007; Lowe *et al.*, 2005), and the level of both intra- and interspecific sequence variability may differ significantly between lineages (Caron *et al*., 2009). Conversely, morphological criteria are often unsufficient for distinguishing protistan species, and a single

morphospecies may encompass huge cryptic species diversity (Weisse, 2008). Thus, the diversity of free-living eukaryotic microorganisms is still a subject of general debate.

Molecular surveys are also instrumental for decoding the ecological distribution of certain protistan lineages (Massana *et al.*, 2014) and can be combined with culture-based approaches (Berney *et al.*, 2013). Combined studies have a special importance for environmental data interpretation and revealing different types of possible artifacts. However, studies combining both approaches are very rare and little is known about the biogeography of individual lineages of protists in extreme environments including anoxic sites (Stock *et al.*, 2013).

From the ecological point of view, environmental, PCR-based studies indicate a very patchy distribution of phylotypes and tentatively suggest the possibility of a high degree of habitat specialization of protists (Orsi *et al*., 2011). Because many uncultured protistan lineages detected in environmental studies are represented by only a single or a few sequences, the existence of an extremely diverse protistan 'rare biosphere' was proposed (Caron and Countway, 2009). The term 'rare biosphere' should not be considered simply as a synonym for assemblage of rare species. It is rather assemblage of species that are rare in the original community (in dormant or active stage). It was shown that microbial members of the rare biosphere can become abundant in a community after disturbance. Thus, composition of rare biosphere is probably changing in time and members of the rare biosphere can have important roles in maintaining ecosystem processes in different environmental conditions (Caron *et al*. 2012; Sjöstedt *et al*. 2012).

Members of the supergroup Excavata are just rarely found in environmental sequence libraries. Besides, just approximately 2.500 species of Excavata have been described so far (see Adl *et al*., 2007). Namely plesiomorphic lineages (i.e. CLOs, trimastigids, and jakobids) are striking examples of species poverty of the group, because they are known just in a few species

and several strains. From them, only jakobids are frequently found in environmental sequence libraries. Furthermore, jakobids are probably crucial lineage in our understanding the evolution of Excavata and possibly whole Eukaryota.

Jakobids are poorly studied heterotrophic biflagellates. They constitute a deep-branching clade and are divided into two lineages, Andalucina and Histionina. In total, they are comprised of only nine described species, the majority of which have been reported from fresh water; two species, *Jakoba libera* and *Andalucia incarcerata* (= *Stygiella incarcerata* comb. nov.), live in marine environments. The latter species is the only described anaerobic jakobid.

Although jakobids were not recognized as a taxon until the 1990s, and their diversity is understudied, they have recently attracted considerable attention because of their plesiomorphic, bacterial-like mitochondrial genomes (Burger *et al*., 2013). In addition, jakobid cells possess a plesiomorphic arrangement and composition of the flagellar apparatus (Simpson and Patterson, 2001; Yubuki and Leander, 2013).

Recently, Derelle *et al*. (2015) proposed that the root of eukaryotic tree lies between Opimoda and Diphoda group and the last common ancestor of all eukaryotes was probably jakobid-like protist. Derelle *et al*. showed that malawimonads, a small group of heterotrophic nanoflagellates that are almost indistinguishable from jakobids by light and electron microscopy (O´Kelly *et al*., 1999), are not closely related to other Excavata. Instead, they form a clan with Amorphea (Opimoda) whereas jakobids and other excavates belongs to Diphoda.

Although the jakobids have been frequently detected in anoxic habitats by environmental approaches, only two strains of a single species have been cultured so far. A comprehensive phylogenetic analysis including environmental sequences closely related to jakobids has been missing, and the monophyly of jakobids detected in anoxic/microoxic habitats has been unclear (Simpson *et al.,* 2008). Here we cultured 21 new jakobid strains from various marine

anoxic/microoxic habitats worldwide. Subsequently, we compared data from the strains with data obtained from environmental studies. Our results show that anaerobic jakobids constitute a globally distributed clade and are relatively common in anoxic marine environments. This study provides another illustration of how the use of culture-based, or environmental molecular-based approaches alone is insufficient to detect the full extent of species diversity of protists.

EXPERIMENTAL PROCEDURES

Organisms: As detailed in the Supplementary material S1, most of the 21 strains were isolated from marine/brackish coastal sediments; strain LUC3N was obtained from sediment 20 m under the sea surface (see S1 for details). Samples were initially inoculated into the artificial seawater-based ATCC medium 1525 and then subcultured once a week.

Light Microscopy: Morphological observations were performed using a BX51TF Microscope equipped with a DP70 camera (Olympus). Living cells were observed using Differential Interference Contrast. Protargol-stained preparations were prepared following Nie's (1950) protocol as modified by Pánek *et al.* (2014a) and observed by bright-field microscopy. Cell length was measured in 50 cells for each isolate.

Transmission Electron Microscopy: The cell suspension of strain LUC3N with addition of 20% BSA was frozen using the high-pressure freezer (Leica EM Pact II) and then transferred to the freeze substitution unit (Leica EM AFS). The ice in the specimen was replaced by anhydrous acetone containing 2% osmium tetroxide. The sample was embedded in EMbed-812 (EMS) and polymerized at 62˚C for 48 h. The ultrathin sections were stained with uranyl acetate (2%) and lead citrate and examined using a TEM JEOL 1011.

Nucleic acid extraction, PCR Amplification, Sanger and 454 Sequencing: SSU rDNA was amplified from genomic DNA using universal eukaryotic primers (Medlin *et al.*, 1988); the

alpha-tubulin gene of strains LUC3N and PC1 and beta-tubulin gene of strain LUC3N were amplified using universal primers (Edgcomb *et al.,* 2001; Yoon *et al.,* 2008). For details of methods for nucleic acid extraction, PCR amplification, cloning and sequencing see supplementary material S1. Total RNA was extracted from a monoeukaryotic culture of strain LUC3N. Methods used for cDNA library construction, 454 sequencing, cluster assembly, and gene transcript annotation for LUC3N are described in the supplementary material S1. Sequences reported in this study are available in GenBank (KP144389–KP144409; XX-XX). Sequence data from Saanich Inlet have been deposited in GenBank, accession numbers YY-YY.

SSU rDNA Dataset Construction: A final dataset of SSU rDNA genes of all isolates and containing sequences from newly reported strains and cultured and uncultured jakobids from GenBank was constructed as described in the supplementary material. The dataset was aligned using MAFFT 7.110 server (*http://mafft.cbrc.jp/alignment/server/*) with the G-INS-i algorithm (Katoh *et al.*, 2005). The alignment was manually edited in BioEdit 7.0.4.1 (Hall, 1999) and is deposited in the supplementary material S1A.

Protein Datasets Construction: Selected translated amino acid sequences from LUC3N were added to single-protein datasets constructed as described in the supplementary material S1. Amino acid sequences were aligned using the MAFFT 7.110 server at default settings and trimmed manually. To test for paralogs or contaminants, we performed phylogenetic analyses of the alignments (not shown) and checked the trees manually. A final multi-protein dataset (deposited in the supplementary material S1B) was constructed by concatenation of independently aligned single-protein datasets for actin, β-tubulin, cytosolic HSP70, cytosolic HSP90, EF-2, and EF-1α genes. Gene sequences for α-tubulin were analyzed separately.

Phylogenetic Analyses: Phylogenetic trees were constructed in RAxML 8.0.19 (Stamatakis, 2006) using GTRGAMMAI (nucleotides) or PROTGAMMAILG (amino acid residues) models

with 100 maximum likelihood tree searches. Branch support was estimated from 1000 non-parametric bootstrap replicates. PhyloBayes 3.3 (Lartillot and Philippe, 2004) was run on all datasets using CAT GTR (nucleotides) or CAT POI model (amino acid residues). Two or four independent chains were run until their maximum observed discrepancy was < 0.1, and the effective sample size of all model characteristics was at least 100. Consensus trees and posterior probabilities were than calculated using the BP comp program with 25 % of generations discarded as burn-in, and sampling every 10 trees.

Data Mining in Pyrotag Archives: To find jakobid sequences in environmental pyrotag archives, we used the QIIME package 1.7 (Caporaso *et al.*, 2010). Sequences from each dataset were clustered using a 97% cut-off and representative sequences of each OTU_{97} were taxonomically annotated against both the Silva 111 and the PR2-119 databases.

Genetic distances: We computed uncorrected p distances among and within particular isolates using the region corresponding to positions 545–1544 (SSU rDNA) and 1645–1774 (V9) in KP144395 sequence.

RESULTS

General morphology of cultured strains

We established protist cultures from oxygen-poor localities, predominantly from freshwater and marine littoral sediments. **Jakobids were present in 21 of ~200 marine/brackish cultures and were never observed in freshwater cultures (~250 cultures)**. The cells slowly died when exposed to oxygen, suggesting they were anaerobic or microaerophilic. Accordingly, strains LUC3N, BUSSPRAND, and PC1 were successfully grown in an anaerobic chamber for a long period.

The cells morphologically resembled other naked jakobids and formed two morphotypes previously reported in a survey of *Stygiella incarcerata* by Simpson and Patterson (2001): (a) frequently attaching cells with a conspicuous groove (grooved cells) and (b) cells with a less distinct, shortened or narrower groove (swimming cells). Both morphotypes were able to move with a spiraling motion; the swimming cells moved rapidly and usually did not attach to the substrate. Examination of the morphology was complicated due to the variability among cells within a single strain combined with similarity of swimming and grooved cells among strains. Thus, we had to investigate a lot of cells from several culture passages to distinguish between species properly. Dimensions of living and protargol-stained specimens of grooved cells of all species are documented in the supplementary material S2.

Phylogeny of anaerobic jakobids

A comprehensive search of sequence data in GenBank revealed **83 environmental SSU rDNA clones** of jakobids from a variety of marine, hypersaline, and brackish oxygen-poor waters and sediments worldwide (see supplementary material S3). The phylogenetic position of the new strains and environmental clones was determined using maximum likelihood and Bayesian methods (Fig. 1). Similar to previous analyses, jakobids were not recovered as a clade and formed two lineages (Lara *et al.*, 2006; Simpson *et al.*, 2008). The first one, Histionina *sensu* Cavalier-Smith (2013), was highly supported. The second lineage, Andalucina *sensu* Cavalier-Smith (2013), was less supported and contained *Andalucia godoyi*, *Stygiella incarcerata* as well as sequences from 21 new jakobid strains, all environmental clones of jakobids from oxygen poor sites and six clones from the soda lake Nakuru.

Andalucina split into two clades, the first clade, Andaluciidae, contained aerobic *Andalucia godoyi* and six environmental clones of an uncultured lineage from the lake Nakuru.

The second clade, Stygiellidae fam. nov. included all new isolates of anaerobic jakobids, two previously published strains of *Stygiella incarcerata*, and environmental clones of jakobids from oxygen-poor sites. SSU rDNA sequences of Andalucina, including environmental lineages, contained the specific C:G base pair within the basal stem of helix 27 as described by Lara *et al.* (2006); the other jakobids displayed an A:T base pair in the same position.

Stygiellidae split into six statistically well-supported lineages: *Stygiella* gen. nov., *Velundella* gen. nov., and four environmental clades (EC I–IV). The interrelationships among these lineages remained unresolved except for EC II–IV which formed a relatively well-supported clade. Genetic distances among SSU rDNA sequences of Stygiellidae are documented in the supplementary material S5. Monophyly of particular *Stygiella* and *Velundella* species as well as EC I–IV was statistically highly supported; *Stygiella cryptica* sp. nov. and *Velundella nauta* sp. nov. were represented by only a single sequence.

In order to pinpoint relationships among the genera *Velundella, Stygiella* and *Andalucia* more clearly, we carried out a concatenated analysis of six protein-coding genes (Fig. 2). Both Andalucina and Histionina appeared robustly monophyletic; *Velundella* and *Stygiella* constituted a robust clade.

The alpha-tubulin gene was not included in the final multi-protein analysis, because Andalucina and Histionina have strikingly different sequences that possibly represent different paralogues (Simpson *et al.*, 2008). Our phylogenetic analysis is consistent with this assumption since the newly obtained sequences of *Velundella trypanoides* sp. nov. and *Stygiella cryptica* grouped with other members of Andalucina, Trichozoa and opisthokonts; specifically with *S. incarcerata* and *Andalucia godoyi* (see supplementary material S6).

***Stygiella* gen. nov.**

For extended version, see supplementary material S2.

The genus *Stygiella* (Fig. 3A – J; V – AC) included 17 strains, 15 of which were isolated during this study. The strains were morphologically similar to each other, usually 6–9 µm long, crescent-shaped in lateral view, and possessing a broadly open, diamond-shaped groove that occupied either the entire or almost the entire ventral side of the grooved cell, reaching the posterior end. In the SSU rDNA tree, the genus *Stygiella* split into four lineages representing separate species. *S. incarcerata* (6.7–9.5 µm long) consisted of strains with relatively rare swimming cells and grooved cells that usually swam freely.

Stygiella adhaerens sp. nov. (5.0–8.4 µm long) and *S. cryptica* (6.4–10.5 µm) were morphologically very similar to each other and to *S. incarcerata*. Unlike most *S. incarcerata* strains, the grooved cells of both species very often adhered to the substrate by the anterior or the posterior flagellum and swam rarely. *S. cryptica* was the only stygiellid that possessed helix E23/3 in the hypervariable region V4 of SSU rRNA (see supplementary material S2).

In contrast to other *Stygiella* species, grooved cells of *S. agilis* sp. nov. (5.6–8.9 µm long) adhered to the substrate using the cell body (laterally or dorsally) and were somewhat narrower. Grooved cells swam rarely, while swimming cells were extremely abundant and constituted the dominant cell morphotype.

***Velundella* gen. nov.**

For extended version, see supplementary material S2.

The genus *Velundella* (Fig. 3K–R; AD–AG) consists of two species: *V. nauta* and *V. trypanoides*; and environmental sequences closely related to the latter species. Generally, cells of *Velundella*

spp. were conspicuously longer than those of *Stygiella*, usually 9–12 μm. Grooved cells possessed a distinct, spiral groove that did not reach the posterior end of the cell.

V. trypanoides cells (7.8–14.9 μm long) displayed a markedly spiral groove that almost reached the posterior end of the cell. The cells were noticeably elongated when compared with *Stygiella* spp. Grooved cells were broad and possessed a conspicuous groove, while the groove of serpentine-shaped swimming cells was less apparent and narrower. Virtually all grooved cells of *V. trypanoides* were attached to the substrate by the cell body or, sometimes, by posterior cytoplasmic projections.

Velundella nauta (8.4–11.8 μm long) possessed a less spiral and somewhat shorter groove than *V. trypanoides*. The majority of grooved cells were attached to the substrate by flagella, predominantly by the anterior one. The swimming cells possessed a distinctly shortened, narrow groove and sometimes also a bulbous protrusion at the posterior pole of the cell. Unlike the grooved cells, where the posterior flagellum of swimming cells exited the cell at its posterior pole, the posterior flagellum of the swimming cells left the shortened groove at a position approximately ½ the cell length.

Transmission electron microscopy of *Velundella trypanoides* strain LUC3N (Fig. 3S–U) showed that mitochondrion-related organelles lacked cristae and lay close to the nucleus. The single nucleus with central nucleolus contained peripheral heterochromatin, and the posterior flagellum bore a single vane on its dorsal side (up to at least 550 nm in diameter at its broadest).

Pyrotags and their species affiliation

Aside from 83 environmental SSU rDNA clones (see above), we identified **629 stygiellid sequences (25 OTU_{97}; see supplementary material S8)** after exhaustive data mining from anoxic/microoxic marine pyrotag archives from 145 samples from Cariaco Basin, Framvaren

Fjord, Saanich Inlet, and three DHABs: Urania, Discovery and L'Atalante (Bernhard *et al.*, 2014; Hallam *et al*. XXX, Edgcomb *et al*., 2011b; Stoeck *et al*., 2009, 2010). These libraries together contained ~1,000,000 protistan sequences of the SSU rDNA hypervariable region V9, ~277,000 protistan sequences of the V4 region, and ~2,385,000 partial SSU rDNA sequences of V6–V8 regions of all three domains of life including 3,402 eukaryotic reads. The total number of sequences in each species/EC recovered with each method is presented in Table 1.

The only sequence of Stygiellidae found among **V4 sequences** was 273 bp long and differed by three substitutions from sequences EF526735, DQ310256 and EF526837 (all of them represent EC I). Pyrotags of the **V9 region** were not longer than 210 bp, and their taxonomic affiliation was difficult to determine. Initially, we performed phylogenetic analysis of the V9 region including all available stygiellid sequences (see supplementary material S8). Genus *Stygiella* as well as EC II and IV were highly supported, while statistical support for other species was low. Therefore, we were able to determine species affiliation of only 233 pyrotags belonging to EC II or IV (five OTU_{97}) and 97 pyrotags of *Stygiella* spp. (four OTU_{97}). The species affiliation of other V9 pyrotags representing 14 different OTU_{97} remained unresolved. Conversely, we were able to assess species affiliation of almost all pyrotags by comparison of genetic distances among and within stygiellid species/environmental clades. Genetic distances between V9 pyrotags and sequences of particular species were lower or only slightly higher than intraspecific distances between sequences with clear species affiliations (see supplementary material S5). Only one group of four OTU_{97} (26 pyrotags) was not assigned to a particular species/EC using this approach. This group is referred here to as EC V. The V6-V8 regions read library contained 56 jakobid reads (ca 480 bp in length). All of them represented a single genotype that belongs to EC IV and differed in a single nucleotide from the sequence EF526978.

Distribution and salinity tolerance of Stygiellidae

Stygiellidae have been found in marine, oxygen-poor habitats, often in the presence of sulfides, ammonium or methane (see supplementary material S3). However, sequences belonging to EC IV have also been reported from oxic waters of Saanich Inlet ($c0_2$ > 100 µM) with non-detectable amounts of sulfide and ammonium (S. Hallam, unpubl.). All natural localities where stygiellids were detected by traditional or environmental approaches, are summarized in Fig. 4.

To reveal differences in salinity tolerance among and within species of Stygiellidae, as well as their ability to survive in freshwater habitats, we examined five monoeukaryotic cultures of *Stygiella incarcerata*, *S. agilis* and *Velundella trypanoides*. None was able to grow in salinity corresponding to fresh water. We also detected differences in salinity tolerance among examined species (see Fig. 5).

DISCUSSION

Phylogenetic implications

Our phylogenetic analyses indicate that all jakobids detected in anoxic habitats constitute a clade, the Stygiellidae, that represents a sister lineage to the Andaluciidae, aerobic protists reported from soils and alkaline lakes (Figs. 1, 2). Together, Stygiellidae and Andaluciidae form Andalucina, one of the two jakobid lineages (Burger *et al.*, 2013; Cavalier-Smith 2013; this study). Monophyly of Andalucina is clearly validated by several lines of evidence: (1) the SSU rDNA phylogeny (Fig. 1), (2) the phylogenetic analysis based on six protein-coding genes (Fig. 2), (3) the α-tubulin gene phylogeny (supplementary material S6), and (4) the unique C:G base pair within the basal stem of helix 27 in the SSU rRNA molecule as defined by Lara *et al*. (2006).

Our strains span two of the 6–7 clades that we identified within the Stygiellidae. These two cultured clades are described here as *Velundella* and *Stygiella*. The other stygiellids still

remain uncultured (EC I–V), and their relationship to *Velundella* and *Stygiella* is unresolved. EC II–IV form a monophyletic group, but their close relationship to EC I was not sufficiently supported. The phylogenetic position of EC V is even more uncertain, because only a very short part of its SSU rDNA sequence is available (172 bp). Moreover, it was not possible to compare EC V to EC III because their SSU rDNA sequences do not overlap.

Molecular and species diversity of Stygiellidae

In order to reveal species diversity within Stygiellidae, we determined the species boundaries among our strains using phenotypic characterization in combination with SSU rDNA analysis. This approach indicates that our isolates represent six separate species. As a next step, we assessed species identity of available SSU rDNA sequences obtained by environmental PCR-based approaches. For deep-branching environmental lineages, we established provisional, monophyletic groups referred to here as EC I–IV. Intraspecific genetic distances among SSU rDNA of stygiellids did not exceed 4.6%, and minimum interspecific distance was 6.7%. Minimum SSU rDNA distances between EC I–IV were greater than 6.8%. This was significantly greater than observed intraspecific distances, and is comparable to the distance between particular *Velundella* or *Stygiella* species. We therefore assumed that the four environmental clades represent separate species, and Stygiellidae comprises at least 10 species in total. This means that Stygiellidae contains at least the same number of species that has previously been described for all Jakobida. Stygiellid species are morphologically very similar, but genetically and ecologically diverse. Our data further indicate that particular species of Stygiellidae also differ in salinity tolerance or tendency and style of their attachment to the substrate.

Ecological diversity of Stygiellidae

Stygiellid sequences were found almost exclusively in environmental libraries from anoxic or microoxic sites (see results for references). DNA-based data from Saanich Inlet represent the only exception (S. Hallam, unpubl.), because a single OTU of Stygiellidae was detected not only in anoxic or microoxic samples, but also in oxic waters. This observation could be explained in several ways, e.g. if this taxon is either allochtonous to oxic waters in Saanich, particle associated, and therefore, protected from oxygen exposure, or is delivered into oxic waters due to seasonal turnovers that occur in Saanich Inlet. Moreover, this OTU belongs to the EC IV that has been repeatedly detected in anoxic, sulfide-rich environments in DNA- and RNA-based studies. RNA molecules are thought to better target metabolically active, indigenous members of microbial communities compared to DNA (Stoeck *et al.*, 2007). Stygiellid ribosomal RNA has been reported from habitats with oxygen concentrations up to 19.8 µmol/l. Experimental data from *Stygiella* and *Velundella* also support the anaerobic lifestyle of stygiellids since their mitochondria are acristate, and the cells are not able to grow under oxic conditions (Simpson and Patterson, 2001; this study).

We detected active cells of Stygiellidae in a wide range of marine environments including brackish or hypersaline waters and inland salt springs, but they most likely do not inhabit freshwater sites. This assumption is supported by three independent lines of evidence: (1) their sequences are absent from freshwater clone libraries, (2) isolation of Stygiellidae from freshwater anoxic habitats was unsuccessful, and (3) our strains of Stygiellidae were unable to grow in the freshwater medium.

RNA-based environmental data further suggest an extraordinary tolerance of stygiellids to a wide range of environmental conditions (see supplementary material S3). The EC III is an extreme example, because RNA signatures of this taxon were found in both the lower (3501 m

water depth, 35 MPa pressure, 365 ppt salinity, 3000 µM ammonium, 2900 µM sulfides) and upper (3499 m water depth, 36 ppt salinity, 5.5 µM ammonium, non-detectable sulfides) halocline interface of L´Atalante DHAB (Alexander *et al*., 2009). Interestingly, this species was represented by a single phylotype that belonged to only a few eukaryotic OTUs shared between these two closely located, but ecologically and physiologically distinct habitats. It indicates that EC III is able to tolerate extremes of multiple environmental factors and is among the most tolerant or adaptable eukaryotes. In contrast to EC III, the genera *Velundella* and *Stygiella* thrive in salinities ranging from 19 to 74 ppt, or 19 and 56 ppt, respectively. While the observed ecophysiological variability of the strains we examined may be influenced by the presence of different species of bacterial prey in their respective cultures (various undefined species), the same level of salinity tolerance of the three *V. trypanoides* strains examined suggests that the impact of bacterial prey is probably not the main driving factor.

Stygiellidae flourish in sulfidic environments and constitute an important component of eukaryotic communities in anoxic and sulfidic marine habitats. This is shown by both, DNA- and RNA-based environmental studies from Gotland Deep (Stock *et al.*, 2009; Weber *et al.*, 2014). In terms of clone abundance, stygiellids constituted a major component of eukaryotic communities in anoxic, sulfidic layers of Gotland Deep, where samples dominated by stygiellids were collected from number of different sampling sites during two different years. Additionally, stygiellids were frequently detected in marine anoxic habitats using not only molecular, but also culture-based approaches (see results and supplementary material S3).

Sulfide-rich, anoxic Framvaren Fjord (Norway) and Cariaco Basin (Venezuela) provide ideal model systems for studying biodiversity of anaerobic protists including stygiellids; both localities are permanently anoxic, sulfide-rich, and extensive environmental data from a number of sampling sites at each location have been published. When we searched available data sets

from those two locations for stygiellids, we detected the same six species in both geographically distant, but ecologically similar habitats (Fig. 4). In addition, we showed that different stygiellids often co-occur in a single microhabitat. A striking example of this is the F2 sampling site in Framvaren Fjord (Stoeck *et al.*, 2009), where DNA sequences of five different stygiellid species were detected. Our culture-based data further support co-occurrence of stygiellids, since different species or genera were cultured from a single sample, ca 2 ml of sediment (NORMAR/NORMAR2; COORONG/COORONG2), and the original culture of EVROS1 contained two different phylotypes of *Stygiella incarcerata* (EVROS1I, EVROS1T).

Culture-based versus culture-independent methods of diversity detection

Clone and pyrotag DNA-based libraries from anoxic marine habitats are typically dominated by alveolates, stramenopiles, and rhizarians (Edgcomb *et al*., 2011b; Orsi *et al*., 2012; Stoeck *et al*., 2010) and contain relatively small portions of sequences affiliated to Excavata. In fact, environmental studies do not significantly contribute to unveiling species diversity of the excavates, except for euglenozoans and jakobids (Orsi *et al*., 2011; this study). This is surprising because culture-based approaches consistently reveal novel species or even deep-branching lineages of obligatorily anaerobic marine excavates (Kolisko *et al*., 2010; Pánek *et al*., 2014a, 2014b; Yubuki *et al.*, 2010).

To reveal the full diversity of species of anaerobic jakobids and to compare the efficiency of culture-based and culture-independent techniques, we analyzed sequences obtained by three methodologically different approaches: (1) cell culturing and subsequent DNA sequencing; (2) direct extraction of nucleic acid from the environment, amplification and clone sequencing; and (3) next generation sequencing (NGS) of amplified DNA obtained from the environment.

Results of both molecular-based environmental approaches correspond to each other regarding the most abundant jakobid species in nature: EC I, IV and *Stygiella incarcerata*. NGS methods also detect a high abundance of EC II. From those, only *S. incarcerata* is represented in our culture collection and constitutes 43 % of the strains (the most abundant cultured species). Interestingly, the other five cultured species represent together only 1.4 % of stygiellid environmental sequences (Tab.1).

Culture-based approaches have been able to span the entire species diversity of two identified Stygiellidae clades (*Stygiella* and *Velundella*) including two species that have never been detected by culture-independent approaches: *V. nauta* and *S. cryptica*. Short read archives do not contain any *Stygiella* and *Velundella* species but *S. incarcerata* and *V. trypanoides*. By contrast, culture-based approaches were unable to detect any of Stygiellidae environmental clades revealed by other methods (EC I-V). Discrepancies between molecular- and culture-based results are likely at least in part attributable to biases of PCR primers as well as biases of selective culture media and conditions. Based solely on the environmental data, one could hypothesize that at least four of *Stygiella* and *Velundella* species belong to the 'rare biosphere'. Conversely, our culture-based data weaken this hypothesis for *S. agilis* and *S. adhaerens*, since we obtained three cultures of each species. Generally, we can interpret such discrepancy between culture-based and environmental approaches in three different ways: **(1)** If taxa are present at very low abundances in otherwise diverse environmental samples, it is possible that sequencing depth will not be great enough to detect them. In other words, they are common in anoxic localities worldwide in terms of presence, but rare in terms of local abundance. Culture-based approaches may therefore be more efficient in detection of this particular species, especially if low abundance of the species is combined with primer or sampling method biases. We consider such scenario plausible since it is quite easy to establish cultures of certain protistan species even from the single cell (e.g. Hess *et*

al., 2006). **(2)** These species are globally distributed, extremely rare or even missing from many anoxic habitats, but locally abundant under specific conditions. Culture-based methods would detect such species more frequently, as more localities with conditions suitable for growth of the species were explored. **(3)** These species are quite common, their absence in environmental studies is an artifact of primer or sampling method biases. However, sampling method bias is less plausible in the case of *S. agilis*, which only rarely attaches to the substrate and should be easily detectable in the sample. Sequences of all cultured species are compatible at least with some commonly used primer sets in environmental, PCR-based studies (e.g. Behnke *et al*. 2006; Edgcomb, *et al*., 2011; Orsi *et al*. 2012), but nothing is known about the number of rDNA copies in their genomes.

Despite inability of environmental, PCR-based approaches to discover full diversity of stygiellids, we showed that these methods are relatively powerful for revealing the full species diversity of the anaerobic jakobids, since they detected ~80% of the known species diversity of the group. Thus, Stygiellidae seems to be a suitable model group for studying the biogeography and role of bacterivorous nanoflagellates in marine anoxic communities using these approaches. Interestingly, culture-based approaches were more successful in revealing species diversity of two particular subclades, genera *Stygiella* and *Velundella*.

Our data suggests that anaerobic jakobids are much more diverse than previously expected, although its overall diversity is relatively low (tens rather than hundreds of species). Some stygiellid species or environmental clades are very common in the nature (e.g. *Stygiella incarcerata*). By contrast, *Velundella nauta* or *Stygiella cryptica* belong to the 'rare biosphere'.

The ratio of diversity of anaerobic jakobids detected in environmental sequence libraries to the total number of detected jakobid species is inconsistent with data from CLOs (free-living relatives of diplomonads), where environmental studies have been unsuccessful in revealing most

of the diversity described by culture-based methods (Kolisko *et al.,* 2010). This likely reflects biases of the most common PCR primers used in those surveys against CLOs. It is therefore necessary to combine environmental data with data from classical, culture-based approaches to obtain the fullest representation of species, particularly when examining taxonomic groups that may be largely missed by current PCR-based approaches.

Taxonomic summary

Type material consists of protargol preparations deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic. Catalogue numbers are given for each species. For additional information see supplementary material S7.

Stygiellidae fam. nov. Description: Aloricate marine jakobids with acristate mitochondria. Type genus: *Stygiella* gen. nov. Zoobank registration: urn:lsid:zoobank.org:act:4A238129-B037-4B4E-9D15-E205E7B13605. ***Stygiella* gen. nov.** Description: see results. Type species: *Jakoba incarcerata* Bernard Simpson & Patterson, 2000 (= *Stygiella incarcerata* comb. nov.). Zoobank registration: urn:lsid:zoobank.org:act:9EA9ADF7-11C7-40D7-925F-7845D48420C0. ***Stygiella adhaerens* sp. nov.** Description: see results. Syntype: 12/96 (PETROCHORI). Zoobank registration: urn:lsid: zoobank.org:act:E2EF3439-3683-42F1-8985-5A550683F374. ***Stygiella agilis* sp. nov.** Description: see results. Syntype: 9/30-32 and 10/99, 100 (AND). Zoobank registration: urn:lsid:zoobank.org:act:A0114EE7-55D7-4960-8B92-E1AC20019774. ***Stygiella cryptica* sp. nov.** Description: see results. Syntype: 5/97 and 6/51, 52 (PC1). Zoobank registration: urn:lsid:zoobank.org: act:21C5B523-3A4E-405C-A7B4-6507E31F3C1B. ***Velundella* gen. nov.** Description: see results. Type species: *Velundella trypanoides* sp. nov. Zoobank registration: urn:lsid:zoobank.org:act:9472415A-259E-484C-83B2-E0D3B273CD32. ***Velundella trypanoides* sp. nov.** Description: see results. Syntype: 6/27, 28, 84, 85 (LUC3N).

Zoobank registration: urn:lsid:zoobank.org:act:3879E18C-D7F9-4D48-B0B4-EEF635D39664.

***Velundella nauta* sp. nov.** Description: see results. Syntype: 9/92, 93 (BMAND). Zoobank registration: urn:lsid:zoobank.org:act:3879E18C-D7F9-4D48-B0B4-EEF635D39664.

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Supporting information

Additional supporting information (**S1 – S8**) may be found in the online version of the article.

Legends to tables and figures

Figure 1. Phylogenetic tree of Jakobida based on small subunit ribosomal rDNA. The tree is based on alignment of 1588 nucleotide positions and 123 OTUs. The topology was constructed in RAxML using maximum likelihood (GTRGAMMAI model). The values at nodes represent RAxML bootstraps/PhyloBayes posterior probabilities. The values lower than 50% or 0.5 are marked by "-". Clades supported by bootstrap/posterior probability higher than 95/0.95 are marked by thick branches. Sequences from newly isolated strains are in bold. Environmental sequences are represented by their GenBank accession numbers only.

Figure 2: Phylogenetic tree of eukaryotes based on concatenation of six protein-coding genes: actin, β-tubulin, EF1α, EF2, HSP70, HSP90. The tree is based on alignment of 3200 amino acid positions and 47 taxa. The topology was constructed in RAxML using maximum likelihood (PROTGAMMAILG model with four partitions). PhyloBayes was run under CAT POI model. The values at nodes represent RAxML bootstraps/PhyloBayes posterior probabilities. The values lower than 50% or 0.5 are marked by "-". Clades supported by bootstrap/posterior probability higher than 95/0.95 are marked by thick branches.

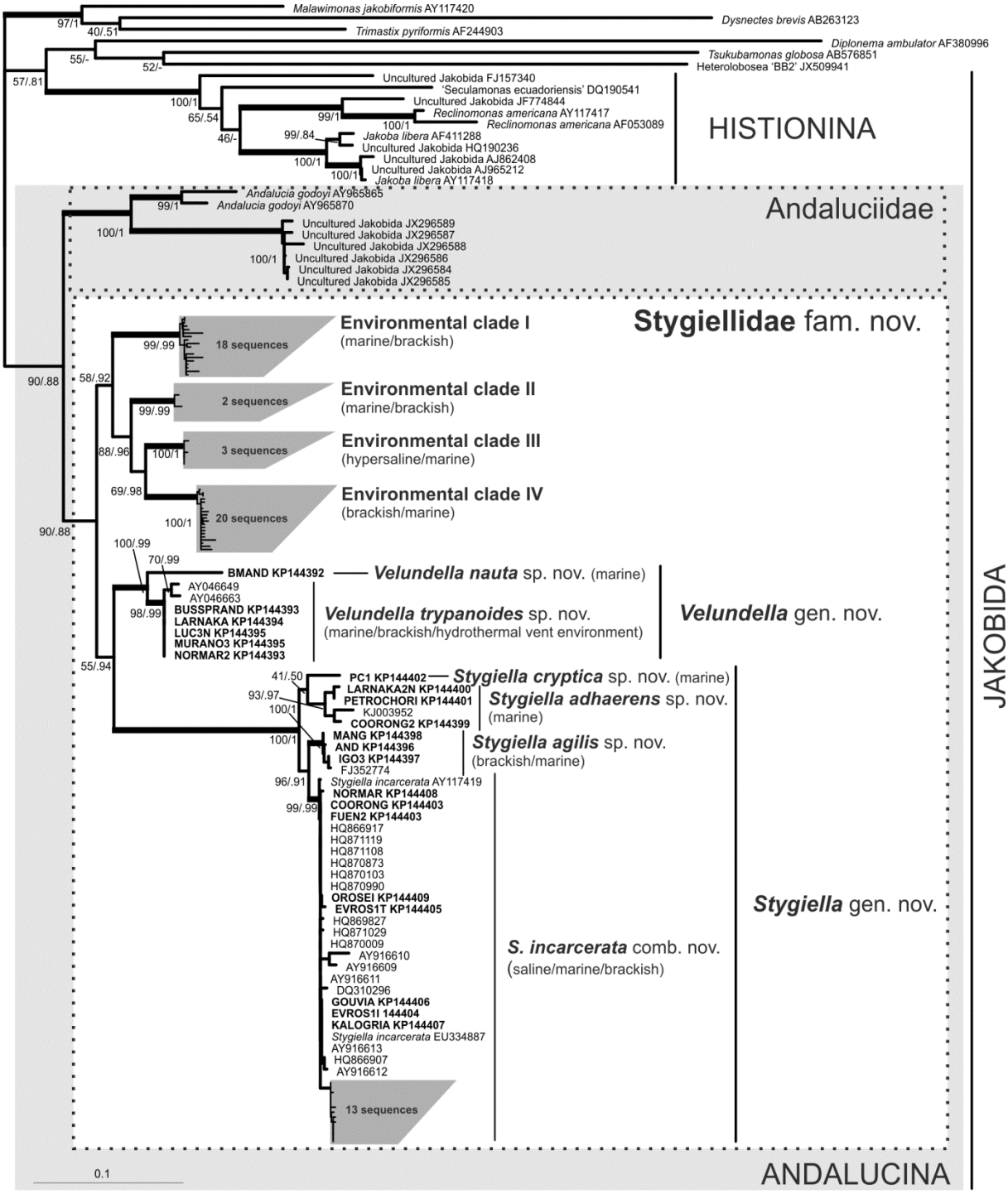
Figure 3. Morphology of Stygiellidae fam. nov. Living cells observed using Differential Interference Contrast (A – R); protargol-stained cells observed by bright-field microscopy (V – AG); TEM micrographs (S – U). Most cells presented here are grooved cells, only a few are swimming cells (J, M, R). Species and strains are arranged as follows: *Stygiella adhaerens* sp.

nov. strain COORONG2 (A, B) and PETROCHORI (Z, AA); *Stygiella incarcerata* comb. nov. strain NORMAR (C, E, W), GOUVIA (D), and EVROS1I (V); *Stygiella cryptica* sp. nov. strain PC1 (F, X, Y); *Stygiella agilis* sp. nov. strain AND (G, J, AC), IGO3 (H, I), and MANG (AB); *Velundella trypanoides* gen. et sp. nov strain LUC3N (K, L, S – U, AD, AE) and BUSSPRAND (M); *Velundella nauta* sp. nov. strain BMAND (N – R; AF, AG). Bar = 5 μm (A – R, V – AG), 500 nm (S), and 200 nm (T, U). Labels – F1: axoneme of the posterior flagellum possessing single dorsal vane; F2: axoneme of the anterior flagellum; M: mitochondrion-related organelle without cristae; N: nucleus; Nu – nucleolus; arrow: anterior flagellum attached to the substrate; double-arrow: cytoplasmic projections or pseudopodia on a cell posterior of Stygiellidae or a bulbous protrusion on cell posterior of *V. nauta* sp. nov.

Figure 4. Geographical distribution of the family Stygiellidae as revealed by environmental, PCR-based approaches and culture-based methods.

Figure 5. Salinity ranges for growth of three species (*Stygiella incarcerata, S. agilis*, and *Velundella trypanoides*). The line thickness indicates relative cell density in the culture; dash line indicates presence in very low densities accompanied by inability of active growth.

Table 1. Number of revealed sequences in each species/EC depending on method used. Total number of protistan (V9 and V4 region of SSU rDNA) or eukaryotic (V6-V8 region of SSU rDNA) pyrotags is indicated in parentheses.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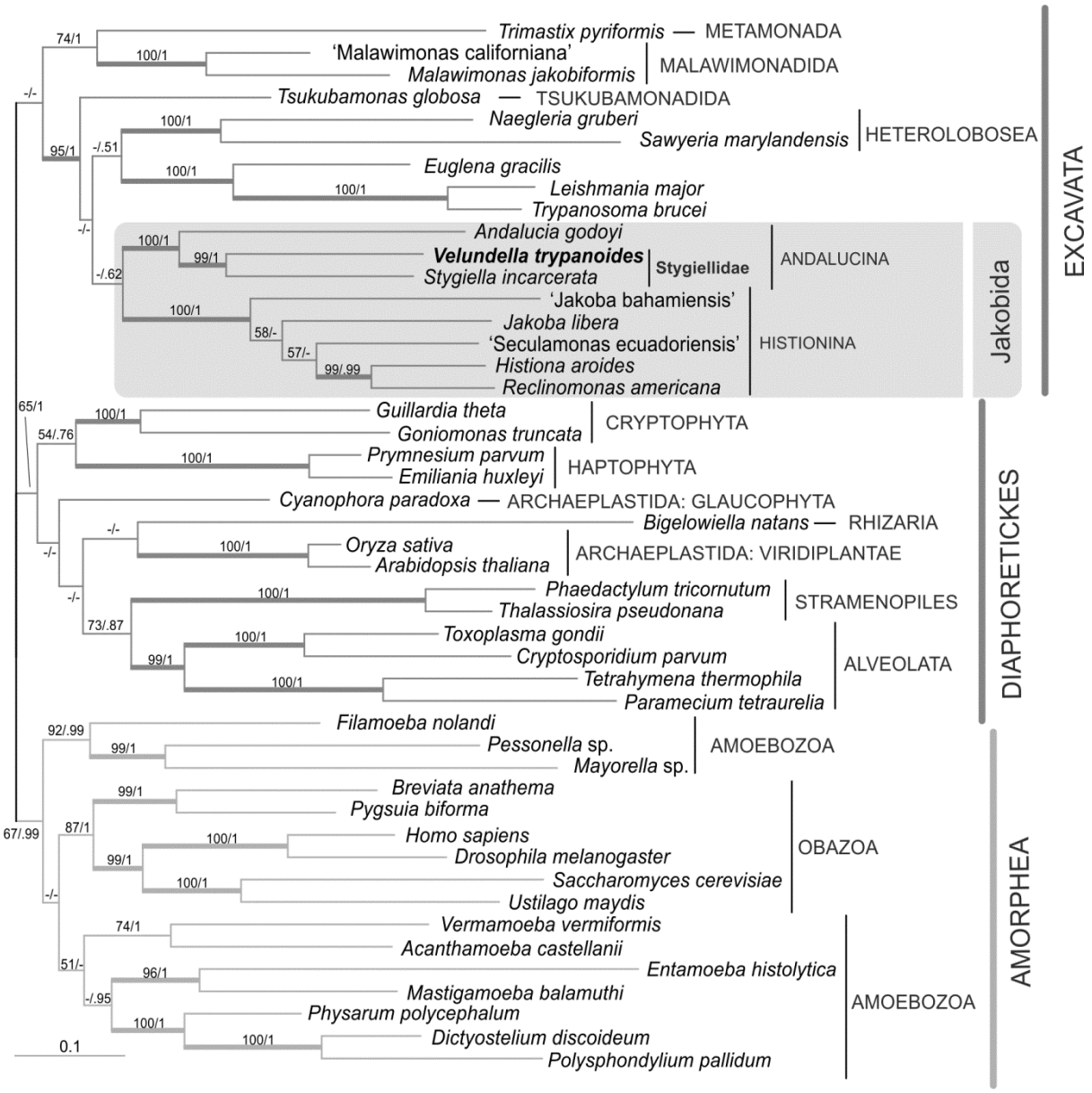
Trimastix pyriformis AF244903
Diplonema ambulator AF380996
Tsukubamonas globosa AB576851
Heterolobosea 'BB2' JX509941
Uncultured Jakobida FJ157340
'Seculamonas ecuadoriensis' DQ190541
Uncultured Jakobida JF774844
Reclinomonas americana AY117417
Reclinomonas americana AF053089
Jakoba libera AF411288
Uncultured Jakobida HQ190236
Uncultured Jakobida AJ862408
Uncultured Jakobida AJ965212
Jakoba libera AY117418
HISTIONINA
Andalucia godoyi AY965865
Andalucia godoyi AY965870
Uncultured Jakobida JX296589
Uncultured Jakobida JX296587
Uncultured Jakobida JX296588
Uncultured Jakobida JX296586
Uncultured Jakobida JX296584
Uncultured Jakobida JX296585
Andaluciidae
Stygiellidae fam. nov.
18 sequences
Environmental clade I
(marine/brackish)
2 sequences
Environmental clade II
(marine/brackish)
3 sequences
Environmental clade III
(hypersaline/marine)
20 sequences
Environmental clade IV
(brackish/marine)
BMAND KP144392
Velundella nauta sp. nov. (marine)
AY046649
AY046663
BUSSPRAND KP144393
LARNAKA KP144394
LUC3N KP144395
MURANO3 KP144395
NORMAR2 KP144393
Velundella trypanoides sp. nov.
(marine/brackish/hydrothermal vent environment)
Velundella gen. nov.
PC1 KP144402
Stygiella cryptica sp. nov. (marine)
LARNAKA2N KP144400
PETROCHORI KP144401
KJ003952
COORONG2 KP144399
Stygiella adhaerens sp. nov.
(marine)
MANG KP144398
AND KP144396
IGO3 KP144397
FJ352774
Stygiella agilis sp. nov.
(brackish/marine)
Stygiella incarcerata AY117419
NORMAR KP144408
COORONG KP144403
FUEN2 KP144403
HQ866917
HQ871119
HQ871108
HQ870873
HQ870103
HQ870990
OROSEI KP144409
EVROS1T KP144405
HQ869827
HQ871029
HQ870009
AY916610
AY916609
AY916611
DQ310296
GOUVIA KP144406
EVROS1I 144404
KALOGRIA KP144407
Stygiella incarcerata EU334887
AY916613
HQ866907
AY916612
13 sequences
S. incarcerata comb. nov.
(saline/marine/brackish)
Stygiella gen. nov.
0.1
ANDALUCINA
JAKOBIDA

Figure 1


Trimastix pyriformis — METAMONADA
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MALAWIMONADIDA
Tsukubamonas globosa — TSUKUBAMONADIDA
Naegleria gruberi
Sawyeria marylandensis
HETEROLOBOSEA
Euglena gracilis
Leishmania major
Trypanosoma brucei
Andalucia godoyi
Velundella trypanoides
Stygiella incarcerata
Stygiellidae
ANDALUCINA
'Jakoba bahamiensis'
Jakoba libera
'Seculamonas ecuadoriensis'
Histiona aroides
Reclinomonas americana
HISTIONINA
Jakobida
EXCAVATA
Guillardia theta
Goniomonas truncata
CRYPTOPHYTA
Prymnesium parvum
Emiliania huxleyi
HAPTOPHYTA
Cyanophora paradoxa — ARCHAEPLASTIDA: GLAUCOPHYTA
Bigelowiella natans — RHIZARIA
Oryza sativa
Arabidopsis thaliana
ARCHAEPLASTIDA: VIRIDIPLANTAE
Phaedactylum tricornutum
Thalassiosira pseudonana
STRAMENOPILES
Toxoplasma gondii
Cryptosporidium parvum
Tetrahymena thermophila
Paramecium tetraurelia
ALVEOLATA
DIAPHORETICKES
Filamoeba nolandi
Pessonella sp.
Mayorella sp.
AMOEBOZOA
Breviata anathema
Pygsuia biforma
Homo sapiens
Drosophila melanogaster
Saccharomyces cerevisiae
Ustilago maydis
OBAZOA
Vermamoeba vermiformis
Acanthamoeba castellanii
Entamoeba histolytica
Mastigamoeba balamuthi
Physarum polycephalum
Dictyostelium discoideum
Polysphondylium pallidum
AMOEBOZOA
AMORPHEA
0.1

Figure 2

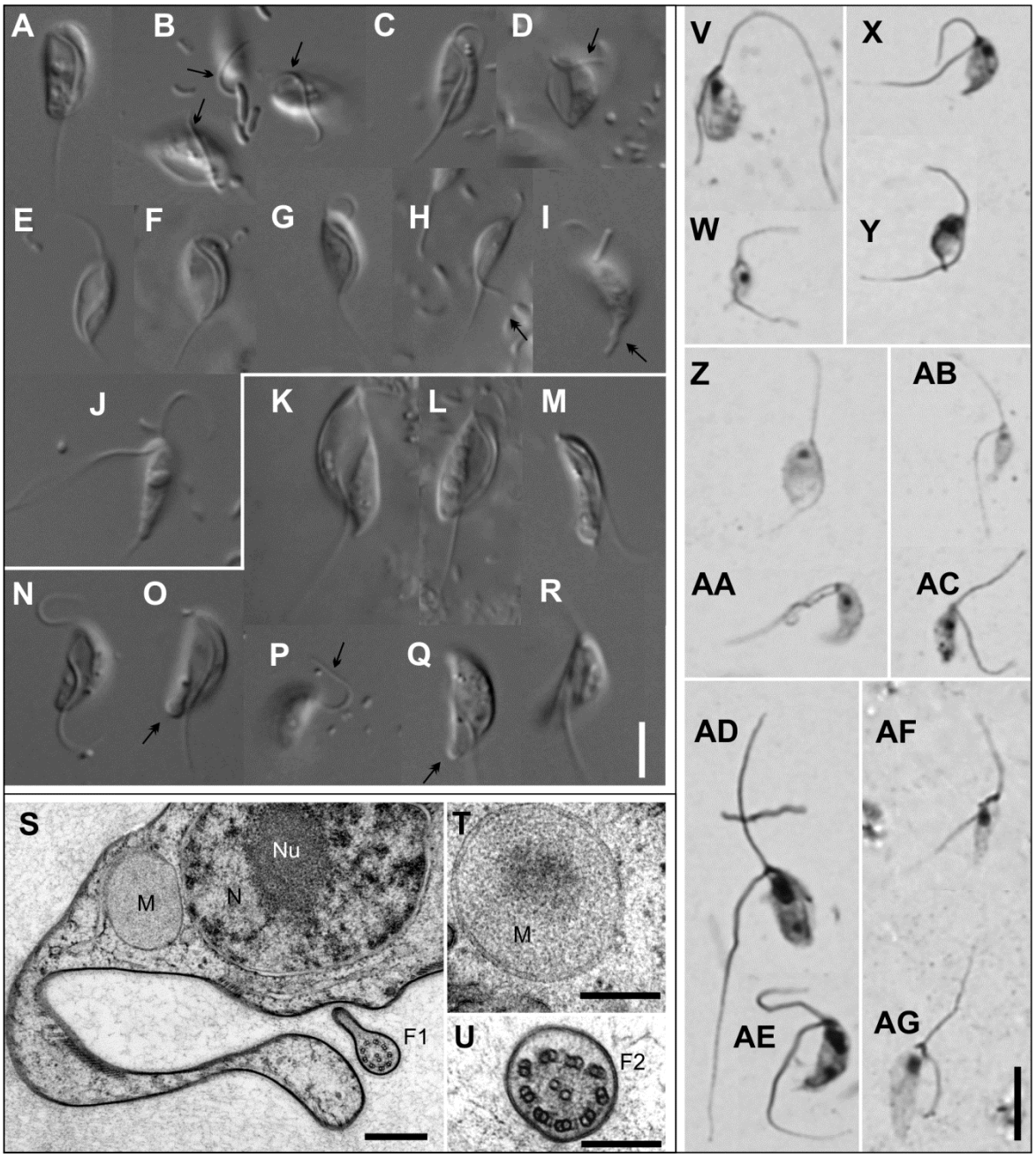

Figure 3

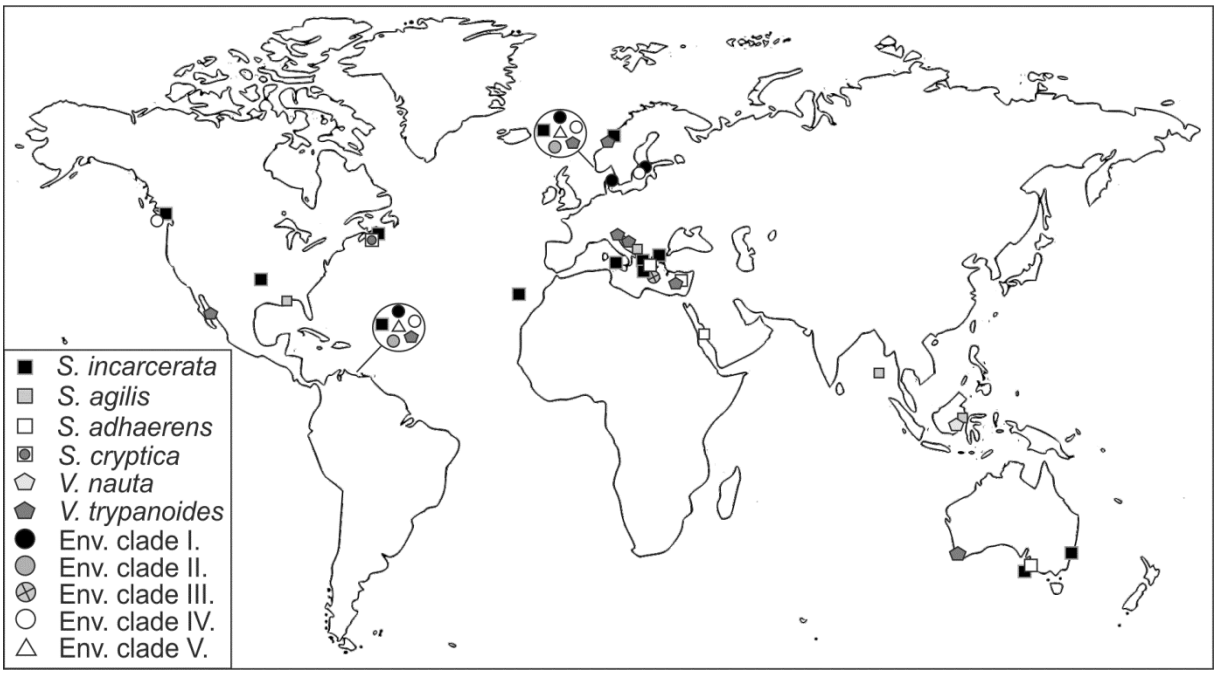

Figure 4

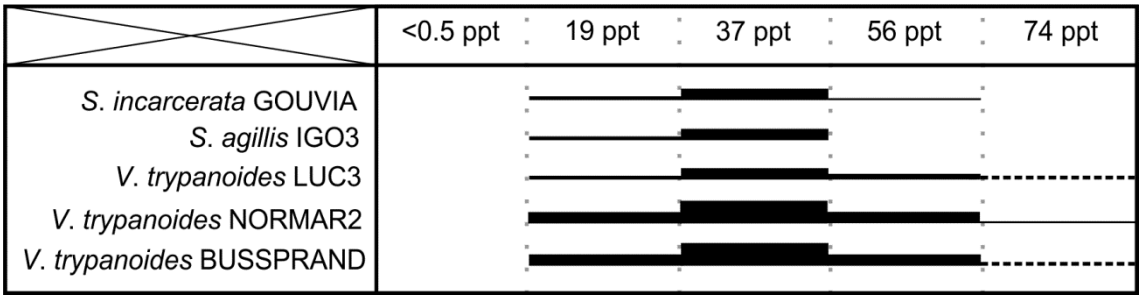

Figure 5

SUPPLEMENTARY MATERIAL S1

LIST OF STRAINS INCLUDED IN THE STUDY

Strains were isolated between 2008 and 2012 and were maintained in polyxenic agnothobiotic cultures with unidentified bacteria at room temperature. They were subcultured once a week. Most cultures were not monoeukaryotic and contained various other protists besides the jakobids (see the table S1.1 below). Strains EVROS1T and EVROS1I (both *S. incarcerata* comb. nov.) were successfully separated and cultured individually after several tens of initial passages. Strains COORONG (*S. incarcerata* comb. nov.) and COORONG2 (*S. adhaerens* sp. nov.) were isolated from a single sample that was inoculated into two tubes containing ATCC medium 1525, and two separate cultures were created. Strains NORMAR (*S. incarcerata* comb. nov.) and NORMAR2 (*V. trypanoides* n. gen., n. sp.) were isolated from the same culture.

Strain	**Locality**	**Coordinates**
***Stygiella incarcerata* (former *Andalucia incarcerata*)**		
COORONG	Coorong NP, Australia	36°04'S, 139°35'E
EVROS1I[1]	Evros delta, Greece	40°48'N, 26°01'E
EVROS1T	Evros delta, Greece	40°48'N, 26°01'E
FUEN2	Punta de Fuencaliente, La Palma, Canary Islands, Spain	28°27'N, 17°50'W
GOUVIA[2]	Corfu Island, Greece	39°38'N, 19°50'E
NORMAR	Nørdre Eidsbukta, Aure, Norway	63 °18'N, 8 °32'E
KALOGRIA[*]	Kalogria, Greece	38°09'N, 21°22'E
OROSEI	Orosei, Sardinia, Italy	40°33'N, 9°68'E
Stygiella adhaerens sp. nov.		
COORONG2	Coorong NP, Australia	36°04'S, 139°35'E
LARNAKA2N[4]	Larnaka, Cyprus	34°51'N, 33°37'E
PETROCHORI	Pilos, Petrochori, Greece	36°58'N, 21°39'E
Stygiella cryptica sp. nov.		
PC1[*]	Peggy's Cove, Canada	44°29'N, 63°55'W
Stygiella agilis sp. nov.		
AND[3]	Elephant beach, Havelock Island, Andaman Islands, India	12°00'N, 92°56'E
IGO3[2*]	Igoumenitsa, Greece	39°31'N, 20°14'E
MANG	Balikpapan Bay, Borneo, Indonesia	1°03' S, 116°42' E
***Velundella nauta* sp. nov.**		
BMAND	Balikpapan, Kalimantan, Indonesia	1°20' S, 116°50' E
***Velundella trypanoides* sp. nov.**		
BUSSPRAND[*]	Marrybrook, Busselton, Australia	33°38'S, 115°11'E
LARNAKA[*]	Larnaka, Cyprus	34°51'N, 33°37'E
LUC3N[*]	Brač island, Croatia	43°17'N, 16°52'E
MURANO3	Murano Islands, Venetian lagoon, Italy	45°27'N, 12°21'E
NORMAR2	Nørdre Eidsbukta, Aure, Norway	63 °18'N, 8 °32'E

Table S1.1. List of strains. Monoeukaryotic strains are marked by asterisks. ([1]) The strain was derived from the same isolate as EVROS1I published by Pánek et al. (2014). ([2]) The strain was derived from the same isolate as GOUVIA published by Pánek et al. (2014). ([3])The strain was derived from the same isolate as AND published by Pánek et al. (2014). ([4])The strain was derived from the same isolate as LARNAKA2 published by Kolisko et al. (2010).

CULTURE MEDIA USED IN THIS STUDY

ATCC 802 freshwater medium (modified)

Add cerophyll (2.5 g) to 1000 ml of distilled water and boil for 5 minutes. Filter through Whatman #1 filter paper. Add 0.5 g Na_2HPO_4 and distilled water to compensate evaporation. Autoclave for 15 – 20 minutes at 121^{o}C.
Store at 4^{o}C.

ATCC 1525 seawater medium (modified)

Solution 1:
Add cerophyll (2.5 g) to 500 ml of distilled water and boil for 5 minutes. Filter through Whatman #1 filter paper. Add distilled water to compensate evaporation. Autoclave for 15 – 20 minutes at 121^{o}C.

Solution 2:
Prepare 300 ml of solution containing NaCl (24.72 g), KCl (0.68 g), $CaCl_2.2H_2O$ (1.36 g), and $MgCl_2.6H_2O$ (4.66 g). Autoclave for 15 – 20 minutes at 121^{o}C.

Solution 3:
Prepare 100 ml of solution containing $MgSO_4.7H_2O$ (6.29 g). Autoclave for 15 – 20 minutes at 121^{o}C.

Solution 4:
Prepare 100 ml of solution containing $NaHCO_3$ (0.18 g). Autoclave for 15 – 20 minutes at 121^{o}C.

When cool, aseptically combine all four solutions. Chlorides, $NaHCO_3$ and sulfide is autoclaved separately to avoid salt precipitation. Store at 4^{o}C.

2X Modified ATCC 1525 medium

Solution 1:
Add cerophyll (2.5 g) to 500 ml of distilled water and boil for 5 minutes. Filter through Whatman #1 filter paper. Add distilled water to compensate evaporation. Autoclave for 15 – 20 minutes at 121^{o}C.

Solution 2:
Prepare 300 ml of solution containing NaCl (49.44 g), KCl (1.36 g), $CaCl_2.2H_2O$ (2.72 g), and $MgCl_2.6H_2O$ (9.32 g). Autoclave for 15 – 20 minutes at 121^{o}C.

Solution 3:
Prepare 100 ml of solution containing $MgSO_4.7H_2O$ (12.58 g). Autoclave for 15 minutes at 121^{o}C.

Solution 4:
Prepare 100 ml of solution containing $NaHCO_3$ (0.36 g). Autoclave for 15 – 20 minutes at 121^{o}C.

When cool, aseptically combine all four solutions. Chlorides, $NaHCO_3$ and sulfide is autoclaved separately to avoid salt precipitation. Store at 4^{o}C.

Cerophyll-based media with salinity range from fresh water to brine water

1) Freshwater: prepared as ATCC 802 freshwater medium (modified)
2) Brackish (19ppt) medium: prepared as 1:1 combination of ATCC 802 freshwater medium and ATCC 1525 seawater medium
3) Normal seawater (37ppt) medium: prepared as ATCC 1525 seawater medium
4) Brine (56ppt) medium: prepared as 1:1 combination of ATCC 1525 seawater medium and 2X ATCC 1525 seawater medium
5) Brine (74ppt) medium: prepared as 2X ATCC 1525 seawater medium

SALINITY RANGES FOR GROWTH

Five different cerophyll-based media with salinity ranging from freshwater to 74 ppt were prepared to determine salinities suitable for growth of monoeukaryotic cultures. 0.25 ml of the culture was inoculated simultaneously into all types of media and examined after 4 and 7 days. Active growth at a particular salinity was confirmed by a transfer into a fresh medium with the same salinity. In order to cut down stress caused by a sharp change of salinity, we established cultures with extreme salinities by inoculation of actively growing cells from cultures with 19ppt and 56ppt salinity. All experiments were done in triplicates.

NUCLEIC ACID EXTRACTION, PCR AMPLIFICATION, SANGER AND 454 SEQUENCING

Genomic DNA was isolated using the ZR Genomic DNA II Kit™ (Zymo Research, USA). Almost complete SSU rRNA gene was amplified using universal eukaryotic primers A and B (Medlin *etal*., 1988). The alpha-tubulin gene of *Velundellatrypanoides* sp. nov. LUC3N and *Stygiellacryptica* sp. nov. PC1 was amplified using nested PCR and two different sets of primers (Edgcomb *etal*., 2001; Yoon *etal*., 2008); for primer sequences see below. The PCR products were purified and directly sequenced or cloned into the pGEM®-T EASY vector (Promega); at least two clones were sequenced.

Total RNA was extracted from cultured cells of *Velundella trypanoides* LUC3N (monoeukaryotic polyxenic culture) using the NucleoSpin RNA II kit (Macherey-Nagel). mRNA was selected with Dynabeads Oligo$(dT)_{25}$ (Invitrogen) and used for cDNA construction by SMARTer PCR cDNA Synthesis Kit (Clontech) following the manufacturer's instructions with the exception of using modified CDS-T22 primer (5'-AAGCAGTGGTATCAACGCAGAGTTTTTGTTTTTTTCTTTTTTTTTTVN-3') instead of 3' BD SMART CDS Primer II A, first strand synthesis time was prolonged to 2 hours, and PCR conditions during second strand synthesis were modified (denaturation: 95°C/ 2 min, amplification: 24 times 95°C/ 10 sec, 65°C/ 30 sec, 72°C/ 3 min, final extention: 72°C/ 5 min). cDNA was normalized by Trimmer cDNA Normalization Kit (Evrogen). Normalized cDNA was used for preparation of sequencing library by Rapid Library Preparation Method (Roche) and sequenced using GS FLX+ chemistry followed by standard analysis of signal processing (454 Life Sciences, Roche). Automatic assembly was performed by Newbler 2.6 software.

ESTs of *Velundellatrypanoides* gen. nov. sp. nov. (strain LUC3N) were screened for previously published *Stygiellaincarcerata* comb. nov. gene orthologues of α-tubulin, β-tubulin, cytosolic HSP70, cytosolic HSP90, EF-2, and EF-1α genes using local BLAST (tBLASTN) in BioEdit 7.0.4.1. The tBLASTN hits were then translated to amino acid residues, using ExPASy SIB Bioinformatics Resource Portal (web.expasy.org/translate/).

SSU rDNA DATASET CONSTRUCTION

Exhaustive BLASTn searches (Altschul *et al.,* 1990) were performed using new and existing Andalucina SSU rDNA sequences to identify all clone sequences from environmental libraries present in the GenBank database (see supplementary material S3). The presence of sequence chimeras was assessed by visual screening of the sequence alignment and subsequent BLASTn searching using contradictory parts of any problematic sequences. This approach identified environmental sequences AY046663, AY916611, AY916612, and AY916613 as chimeras. From chimeric sequences that provided conflicting taxonomic signal (BLASTn searching), we included only the part that was clearly recognized as a part of jakobid SSU rRNA gene.

Preliminary phylogenetic analysis was based on the dataset that contained 83 environmental jakobid clones from anoxic and microoxic samples and several jakobid sequences as

outgroups (phylogenetic tree computed in RAxML is deposited in the supplementary material S4). To the final dataset, we included 123 OTUs with sequences longer than 490 bp: 95 jakobid sequences from GenBank, sequences of 21 new strains of Stygiellidae fam. nov., and 6 eukaryotic sequences from GenBank as outgroup. The final dataset contained 1588 aligned nucleotide positions (**supplementary material S1A**, see below).

PROTEIN DATASETS CONSTRUCTION

Inferred Amino acid sequences obtained from *Velundellatrypanoides* LUC3N were added to single-gene protein datasets containing sequences adopted from seed datasets published by Brown *et al.*, 2013 and Yabuki *et al.*, 2014, or sequences previously deposited in GenBank. To improve taxon sampling of our dataset, we further found genes of interest in ESTs of three different amoebozoans deposited in MMETSP (Keeling *et al.*, 2014): *Mayorella* sp. ATCC 50980, *Filamoebanolandi* ATCC 50430, and *Pessonella* sp. PRA-29.

All amino acid datasets were subsequently aligned with the help of the MAFFT 7.110 server at default settings and tested for undetected paralogs or contaminants using phylogenetic analyses of the alignments. Single-gene trees were checked manually. A final multi-protein dataset contained 3200 aligned characters (amino acid residues) of six protein coding genes: actin, β-tubulin, cytosolic HSP70, cytosolic HSP90, EF-2, and EF-1α. The best partitioning scheme for multi-protein data set was computed in PartitionFinderProtein 1.1.0 (Lanfear *etal*., 2012) under the Bayesian Information Criterion and greedy searching. Final partitioning scheme was based on PROTGAMMAILG model and set as follows: LG, p1 = 1-360, 361-792; LG, p2 = 793-1224, 2013-2586; LGF, p3 = 1225-2012; LG, p4 = 2587-3200. Gene for α-tubulin was analysed separately (for reasons see results). The final multi-protein dataset containing 47 taxa is deposited as **supplementary material S1B** (see below), details on the source sequence data for each taxon is available **as supplementary material S1C** (see below).

PRIMERS USED IN THIS STUDY

Almost complete SSU rDNA was amplified from genomic DNA using primers A and B with an annealing temperature of 50 ^{o}C. The alpha-tubulin gene of *Velundellatrypanoides* LUC3N and *Stygiellacryptica* PC1 was amplified from genomic DNA using nested PCR with annealing temperature of 50 ^{o}C in both PCRs. The primary PCR was carried out with primers ATUB_A and ATUB_B; 0.5 µl of the purified PCR product was used as the template for the secondary PCR using primers ATUB_secF and ATUB_secR. The beta-tubulin gene of *Velundellatrypanoides* LUC3N was amplified from genomic DNA using primers BTUB_A and BTUB_B with annealing temperature of 56.8 ^{o}C

NAME	REFERENCE	PRIMER SEQUENCE (5´- 3´)	TARGET GENE
A	Medlin *etal*., 1988	AYCTGGTTGAYYTGCCAG (F)	SSU rDNA
B	Medlin *etal*., 1988	TGATCCTTCTGCAGGTCCACCTAC (R)	SSU rDNA
ATUB_secF	Yoon *etal*., 2008	TTGTACTGCYTNGARCAYG (F)	Alpha-tubulin gene
ATUB_secR	Yoon *etal*., 2008	ACGTACCAGTGNACRAANGC (R)	Alpha-tubulin gene
ATUB_A	Edgcomb *etal*., 2001	RGTNGGNAAYGCNTGYTGGGA (F)	Alpha-tubulin gene
ATUB_B	Edgcomb *etal*., 2001	CCATNCCYTCNCCNACNTACCA (R)	Alpha-tubulin gene
BTUB_A	Edgcomb *et al*., 2001	GCAGGNCARTGYGGNAAYCA (F)	Beta-tubulin gene
BTUB_B	Edgcomb *et al*., 2001	AGTRAAYTCCATYTCRTCCAT (R)	Beta-tubulin gene

Table S1.2. List of primers used in the study.

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SUPPLEMENTARY MATERIAL S2 - MORPHOLOGY

DIMENSIONS OF STRAINS AND SPECIES

Strain / species	CL LIV	n	CL PTG	n
***Stygiella incarcerata* comb. nov. (= *Andalucia incarcerata*)**				
EVROS1I	7.7 ± 0.4 (7.1 – 8.5) µm	20	4.2 ± 0.5 (3.0 – 4.7) µm	25
FUEN2	7.4 ± 0.5 (7.4 – 9.0) µm	50	N.A.	0
NORMAR	8.2 ± 0.7 (6.7 – 9.5) µm	50	3.9 ± 0.4 (3.2 – 4.7) µm	25
OROSEI2A	7.8 ± 0.5 (6.9 – 9.1) µm	50	N.A.	0
S. incarcerata	7.8 ± 0.6 (6.7 – 9.5) µm	170	4.0 ± 0.5 (3.0 – 4.7) µm	50
Stygiella adhaerens sp. nov.				
COORONG2	7.2 ± 0.7 (6.0 – 8.2) µm	50	4.2 ± 0.4 (3.4 – 5.2) µm	50
LARNAKA2N	6.2 ± 0.7 (5.0 – 8.4) µm	50	N.A.	0
PETROCHORI	7.4 ± 0.5 (6.3 – 8.3) µm	50	N.A.	0
S. adhaerens	7.0 ± 0.8 (5.0 – 8.4) µm	150	4.2 ± 0.5 (3.1 – 5.6) µm	50
***Stygiella cryptica* sp. nov.**				
PC1	8.0 ± 0.8 (6.4 – 10.5) µm	50	4.3 ± 0.6 (3.1 – 5.6) µm	50
***Stygiella agilis* sp. nov.**				
AND	7.4 ± 0.8 (6.1 – 9.0) µm	50	3.9 ± 0.5 (3.3 – 4.9) µm	20
IGO3	7.0 ± 0.8 (5.6 – 8.3) µm	50	N.A.	0
MANG	7.2 ± 0.6 (5.9 – 8.2) µm	50	3.9 ± 0.5 (3.2 – 4.7) µm	20
S. agilis	7.1 ± 0.7 (5.6 – 8.9) µm	150	3.9 ± 0.5 (3.1 – 4.9) µm	40
***Velundella nauta* sp. nov.**				
BMAND	10.1 ± 0.9 (8.4 – 11.8) µm	50	6.3 ± 1.0 (5.1 – 8.1) µm	27
***Velundella trypanoides* sp. nov.**				
BUSSPRAND	10.1 ± 0.5 (9.3 – 11.9) µm	50	6.6 ± 0.7 (4.4 – 7.5) µm	50
LARNAKA	11.2 ± 1.2 (9.6 – 14.9) µm	50	6.6 ± 0.8 (5.2 – 9.6) µm	50
LUC3N	11.1 ± 1.1 (9.5 – 14.1) µm	50	7.2 ± 1.0 (5.8 – 10.6) µm	50
MURANO3	10.7 ± 1.4 (8.8 – 14.6) µm	50	N.A.	0
V. trypanoides	10.4 ± 1.2 (7.8 – 14.9) µm	200	6.5 ± 1.1 (4.4 – 10.6) µm	150

Table S2.1. Dimensions (in µm) of living and protargol stained specimens of *Stygiella* and *Velundella* strains and species: average of cell lengths ± standard deviation (range). CL – cell length, LIV – living cells, n – number of cells, PTG – protargol-stained cells.

EXTENDED VERSION OF RESULTS – MORPHOLOGY OF SPECIES

Morphology of *Stygiella* spp.

Fifteen strains belonged to the genus *Stygiella*. The strains were morphologically similar to each other (Fig. S2.1), usually 6 – 9 µm long, crescent-shaped in lateral view, and possessed a broadly open, diamond-shaped ventral groove. The groove occupied entire or almost entire ventral side of the grooved cell and reached its posterior end (Fig S2.1L, U, HA). Grooved cells attached to the substrate either by flagella (mostly by the anterior flagellum - Fig. S2.1I, Z, AA) or by the cell body (laterally or dorsally). The mode of attachment was stable within

the strains. The swimming cells were often narrowed from the middle to the posterior end (Fig. S2.1JA).

Seven strains (COORONG, EVROS1I, EVROS1T, FUEN2, GOUVIA, KALOGRIA, NORMAR, and OROSEI) belonged to the previously described species *Stygiella incarcerata* comb. nov. (formerly *Andalucia incarcerata*; Fig. S2.1N – AA). The cells were approximately 7.8 μm long and had an ovoid or elongately ovoid shape (in a ventral view). The length of the anterior flagellum of grooved cells did not exceed the body length except for EVROS1I, where the anterior flagellum of some cells was extremely long, more than twice the cell length. The posterior flagellum of *S. incarcerata* was 1.5 times longer than the cell body. In all strains but OROSEI the grooved cells most often swum freely and adhered only occasionally; most cells of the strain OROSEI were attached. Grooved cells of *S. incarcerata* attached to the substrate by the anterior flagellum. A few cells were observed that were attached to the substrate through the cytoplasmic projections on the posterior end of the cell body (strain GOUVIA). Swimming cells were relatively rare in all examined strains.

Cells of *Stygiella adhaerens* sp. nov. (strains COORONG2, PETROCHORI, and LARNAKA2N; Figs. S2.1A – I) and *Stygiella cryptica* sp. nov. (strain PC1; Figs S1.1J – M) were morphologically identical and very similar to that of *S. incarcerata*. *Stygiella cryptica* was the only stygiellid that possessed distinct helix E23/3 in the SSU rRNA hypervariable region V4 (see Fig. S2.4). The cells were approximately 7.0 μm (*S. adhaerens*) or 8.0 μm (*S. cryptica*) long. Unlike most *S. incarcerata* strains, the grooved cells of both species almost always adhered to the substrate by the anterior or, seldom, posterior flagellum. Swimming cells were rare.

Stygiella agilis sp. nov. (Fig S2.1BA – RA) was approximately 7.1 μm long. Unlike in other *Stygiella* spp., the grooved cells adhered to the substrate by the cell body (laterally or dorsally) and were somewhat narrower. Grooved cells swam rarely, whereas swimming cells were extremely abundant and constituted the major component of the population (Fig. S2.1EA, JA). Sometimes, we observed also cells attached to the substrate by cytoplasmic projections on the posterior end of the body (Fig. S2.1OA, PA). The initial step of the attachment of the grooved cells was mediated by the anterior flagellum. Subsequently, cells attached by the cell body and released the anterior flagellum.

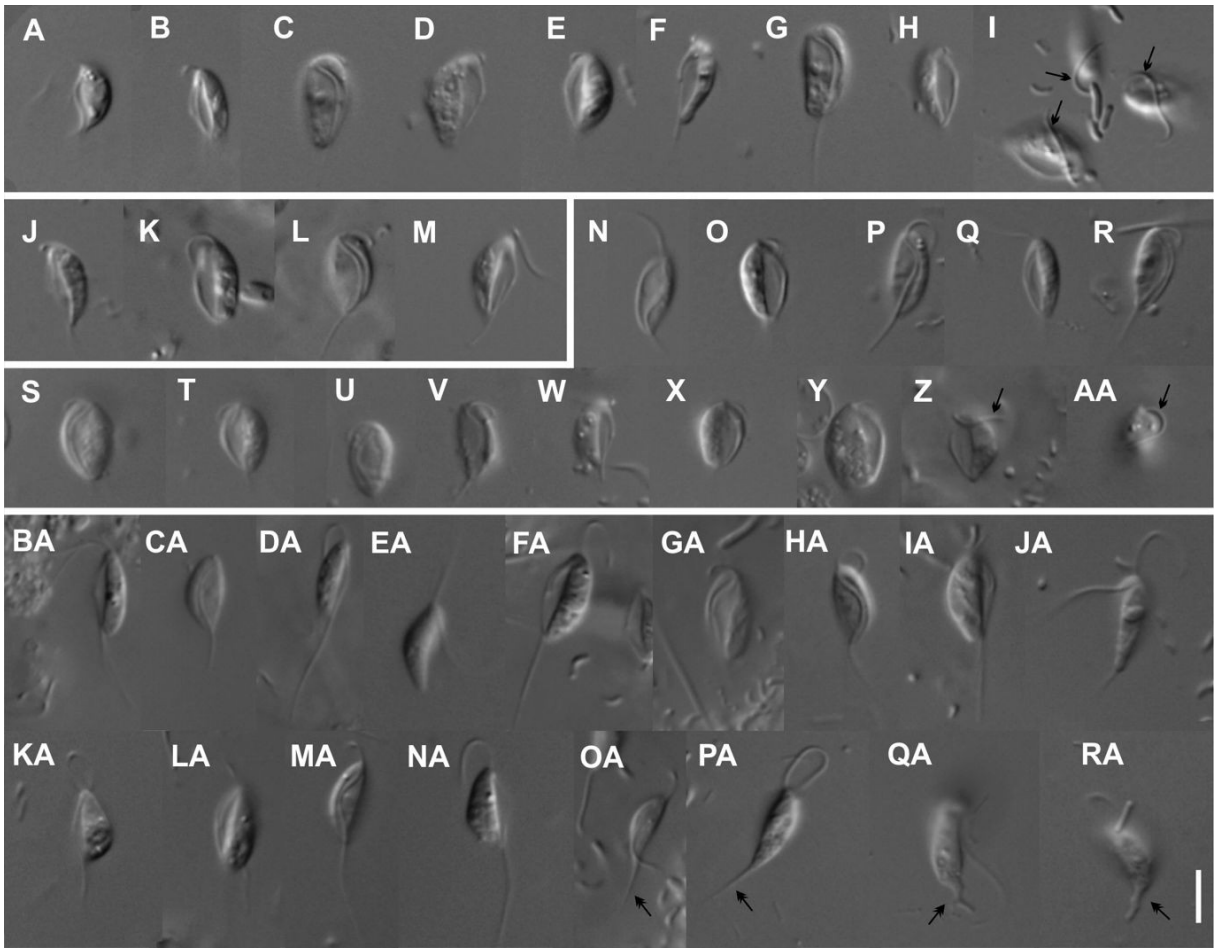

Figure S2.1. Living flagellates of the genus *Stygiella* gen. nov. observed using Differential Interference Contrast. Most cells presented here are grooved cells, only a few cells are swimming cells (EA, JA). Species and strains are arranged as follows: *S. adhaerens* strain PETROCHORI (A – D) and COORONG2 (E – I); *S. cryptica* strain PC1 (J – M); *S. incarcerata* strain NORMAR (N – R), OROSEI (S – Y) and GOUVIA (Z – AA); *S. agillis* strain MANG (BA – EA), AND (FA – JA) and IGO3 (KA – RA). Bar = 5 µm. Arrow: anterior flagellum attached to the substrate; double-arrow: cytoplasmic projections or pseudopodia on cell posterior.

Morphology of *Velundella* spp.

Cells of *Velundella* gen. nov. were usually 9 – 12 µm long. Grooved cells (Fig. S2.2) possessed a conspicuous, more or less spiral feeding groove that did not reach the posterior end of the cell.

Velundella trypanoides sp. nov. (strains BUSSPRAND, LUC3N, LARNAKA, NORMAR2, and MURANO3; Figs. S1.2A – L) displayed markedly spiral feeding groove that almost reached the posterior end of the cell. The cells were noticeably elongated when

compared to *Stygiella* spp. The anterior flagellum was usually shorter than the cell body, and the posterior flagellum was approximately 1.5 – 2 times longer than the cell. Grooved cells were broad and possessed a conspicuous feeding groove, while serpentine swimming cells bore less apparent, narrower groove (Fig. S2.2G). Virtually all grooved cells were attached to the substrate by the cell body or, sometimes, by posteriorly located cytoplasmic projections.

Only a single strain of *Velundella nauta* sp. nov. was cultured and examined (strain BMAND; Fig. S2.2M – T). Both, anterior and posterior flagella were of the same length as in *V. trypanoides*, although the ventral groove was less spiral and somewhat shorter. The posterior pole of the cell sometimes possessed a bulbous protrusion (Fig. S2.2O, P). Most grooved cells were attached by flagella, mainly by anterior ones (Fig. S2.2S, T). Swimming cells possessed distinctly shortened, narrow feeding groove (Fig. S2.2Q). Unlike the grooved cells, where the posterior flagellum of swimming cells exited the cell at its posterior pole (Fig. S2.2N), the posterior flagellum of swimming cells got off the shortened groove approximately in ½ of the cell length (Fig. S2.2Q).

When cultured under harsh conditions (higher concentration of oxygen), rounded forms were observed. The form was markedly similar to putative cysts of *Andalucia godoyi* (Lara et al., 2006; Fig. S2.3). On the other hand, clear cyst wall was observed neither in *A. godoyi*, nor in *V. trypanoides*.

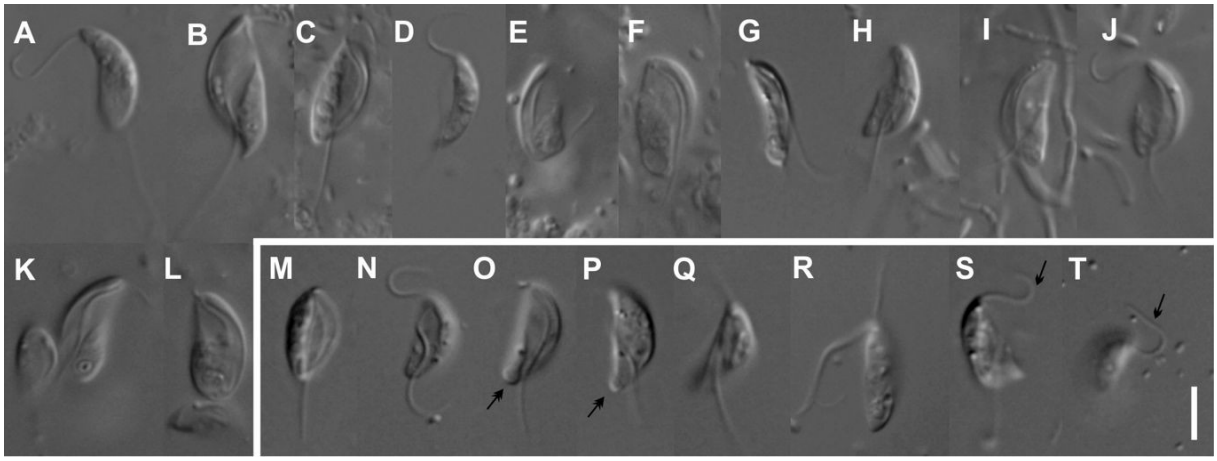

Figure S2.2. Living flagellates of the genus *Velundella* observed using Differential Interference Contrast. Most cells presented here are grooved cells, only a few cells are swimming cells (G, Q). Species and strains are arranged as follows: *V. trypanoides* strain LUC3N (A – C), BUSSPRAND (D – G), LARNAKA (H – J), MURANO3 (K – L); *V. nauta* strain BMAND (M – T). DIC. Bar = 5 μm. Arrow: anterior flagellum attached to the substrate; double-arrow: a bulbous protrusion on cell posterior of *V. nauta*.

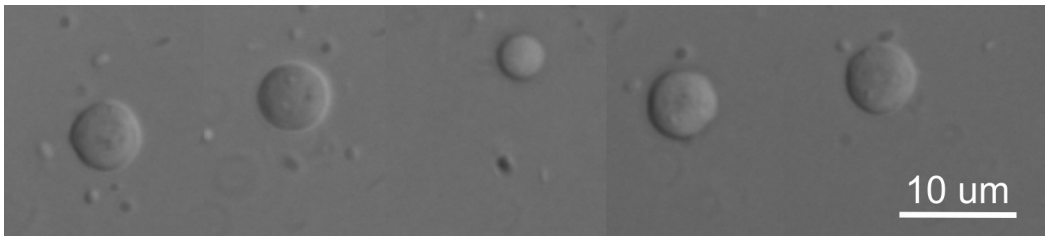

Figure S2.3. Rounded form of *Velundella trypanoides* (strain BUSSPRAND). Cells were observed using DIC.

Figure S2.4 Secondary structure of helices e23/1-3 in SSU rRNA (positions 624-738 in the SSU rRNA of *Stygiella cryptica* strain PC1). Representative sequences from all described species and environmental clades are documented. *Velundella nauta* strain BMT (A); *V. trypanoides* strain LUC3N (B); environmental clade II, DQ310295 (C); environmental clade III, EU446381 (D); *Stygiella agilis* strain AND (E); *S. adhaerens* strain PETROCHORI (F); *S. incarcerata* strain FUEN2 (G); environmental clade IV, FJ153665 (H); *S. cryptica* strain PC1 (I); environmental clade I, EF526837 (J). Structures were reconstructed using MFOLD web server (Zucker, 2003) and VARNA 3.91 software.

Supplementary references

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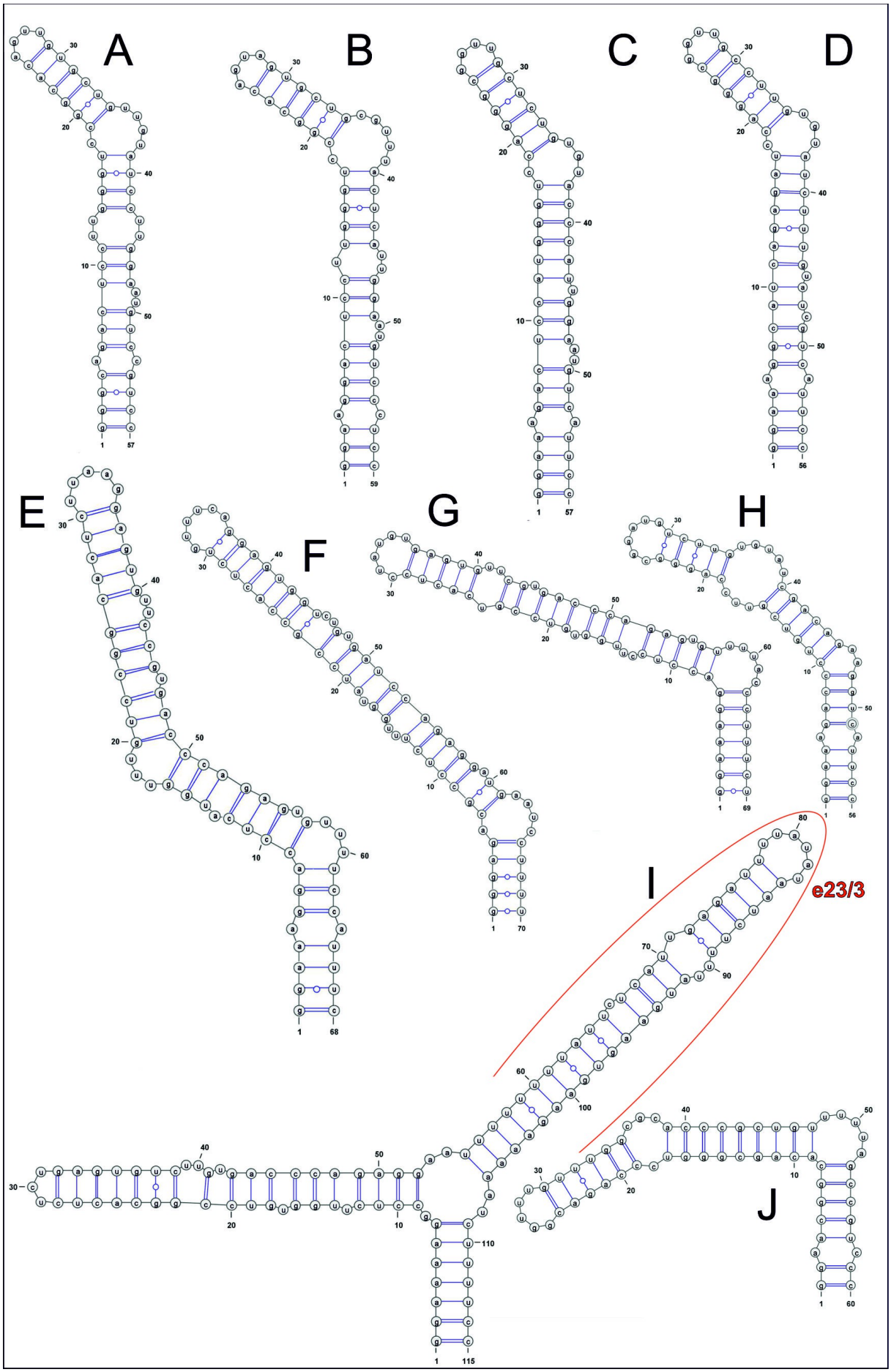
A
B
C
D
E
F
G
H
I
J
e23/3

SUPPLEMENTARY MATERIAL S4 - SSU rDNA PHYLOGENY

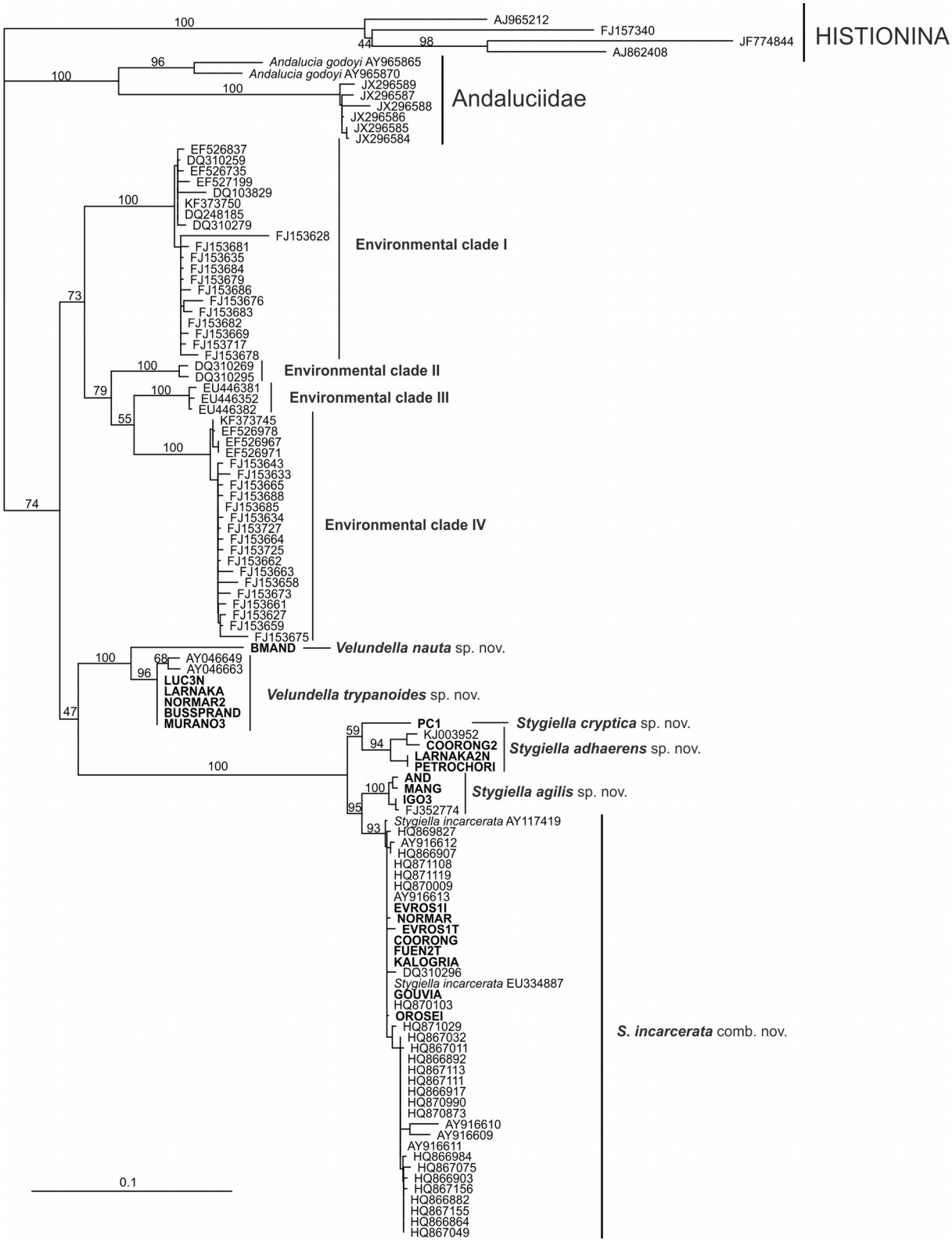

Figure S4.1. Preliminary phylogenetic tree of Stygiellidae based on small subunit ribosomal rRNA (environmental Sanger clones shorter then 490 bp are included). The tree is based on alignment of 1604 positions and 114 OTUs representing all environmental clones of Stygiellidae. Eight andaluciids and four histionids were used as outgroups. The topology was constructed in RAxML using maximum likelihood (GTRGAMMAI model). The values at nodes represent RAxML bootstraps. Sequences from newly isolated strains are in bold. Environmental sequences are represented by their GenBank accession numbers only.

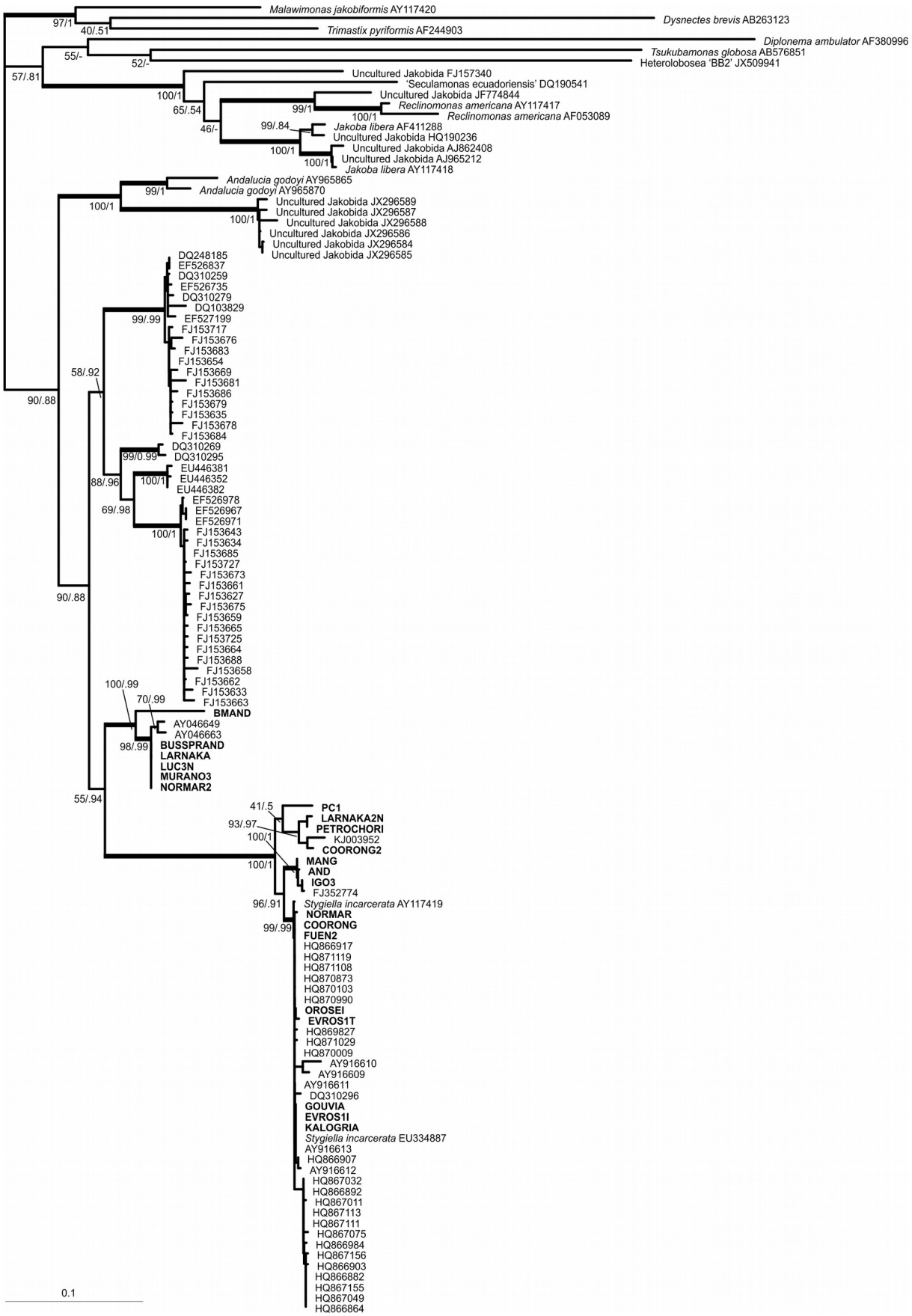
Malawimonas jakobiformis AY117420
Dysnectes brevis AB263123
Trimastix pyriformis AF244903
Diplonema ambulator AF380996
Tsukubamonas globosa AB576851
Heterolobosea 'BB2' JX509941
Uncultured Jakobida FJ157340
'Seculamonas ecuadoriensis' DQ190541
Uncultured Jakobida JF774844
Reclinomonas americana AY117417
Reclinomonas americana AF053089
Jakoba libera AF411288
Uncultured Jakobida HQ190236
Uncultured Jakobida AJ862408
Uncultured Jakobida AJ965212
Jakoba libera AY117418
Andalucia godoyi AY965865
Andalucia godoyi AY965870
Uncultured Jakobida JX296589
Uncultured Jakobida JX296587
Uncultured Jakobida JX296588
Uncultured Jakobida JX296586
Uncultured Jakobida JX296584
Uncultured Jakobida JX296585
DQ248185
EF526837
DQ310259
EF526735
DQ310279
DQ103829
EF527199
FJ153717
FJ153676
FJ153683
FJ153654
FJ153669
FJ153681
FJ153686
FJ153679
FJ153635
FJ153678
FJ153684
DQ310269
DQ310295
EU446381
EU446352
EU446382
EF526978
EF526967
EF526971
FJ153643
FJ153634
FJ153685
FJ153727
FJ153673
FJ153661
FJ153627
FJ153675
FJ153659
FJ153665
FJ153725
FJ153664
FJ153688
FJ153658
FJ153662
FJ153633
FJ153663
BMAND
AY046649
AY046663
BUSSPRAND
LARNAKA
LUC3N
MURANO3
NORMAR2
PC1
LARNAKA2N
PETROCHORI
KJ003952
COORONG2
MANG
AND
IGO3
FJ352774
Stygiella incarcerata AY117419
NORMAR
COORONG
FUEN2
HQ866917
HQ871119
HQ871108
HQ870873
HQ870103
HQ870990
OROSEI
EVROS1T
HQ869827
HQ871029
HQ870009
AY916610
AY916609
AY916611
DQ310296
GOUVIA
EVROS1I
KALOGRIA
Stygiella incarcerata EU334887
AY916613
HQ866907
AY916612
HQ867032
HQ866892
HQ867011
HQ867113
HQ867111
HQ867075
HQ866984
HQ867156
HQ866903
HQ866882
HQ867155
HQ867049
HQ866864
0.1

Figure S4.2. Final phylogenetic tree of Jakobida based on SSU rDNA (all branches retained). The tree is based on alignment of 1588 positions and 123 OTUs. The topology was constructed in RAxML using maximum likelihood (GTRGAMMAI model). The values at nodes represent RAxML bootstraps/PhyloBayes posterior probabilities. The values lower than 50% or 0.5 are marked by „-“. Clades supported by bootstrap/posterior probability higher than 95/.95 are marked by thick branches. Sequences from newly isolated strains are in bold. Environmental sequences are represented by their GenBank accession numbers only.

SUPPLEMENTARY MATERIAL S6 – ALPHA TUBULIN PHYLOGENY

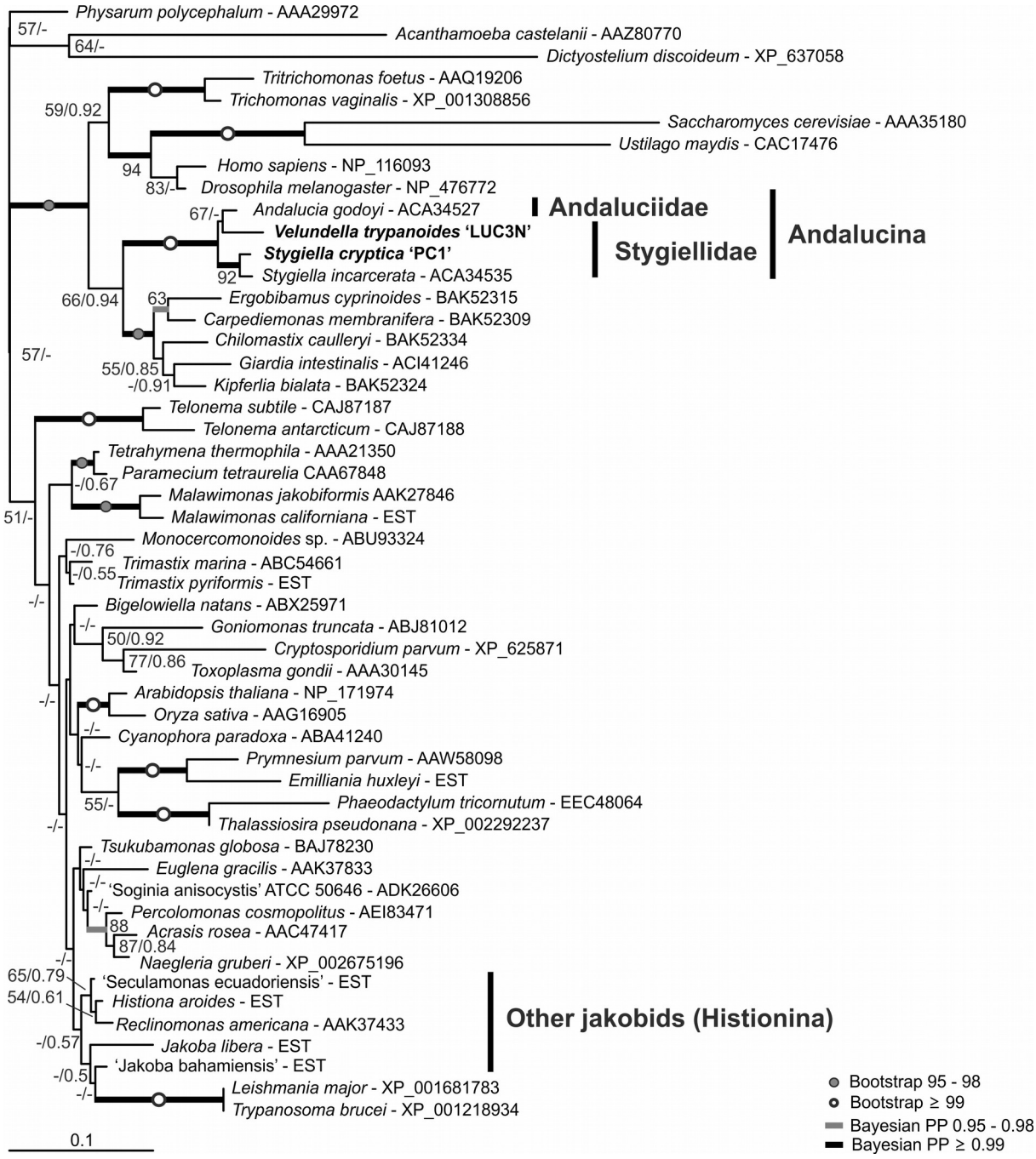

Figure S6.1. Phylogenetic tree of eukaryotes based on α-tubulin. The tree is based on alignment of 405 amino acid residues and 51 taxa. The topology was constructed in RAxML using maximum likelihood (PROTGAMMAILGF model). PhyloBayes was run under POI CAT model. The values at nodes represent RAxML bootstraps/PhyloBayes posterior probabilities. The values lower than 50% or 0.5 are marked by „-".Sequences from newly isolated strains are in bold.

SUPPLEMENTARY MATERIAL S7
FORMAL DESCRIPTION OF NEW TAXA

Excavata: Discoba: Jakobida: Andalucina

Stygiellidae fam. nov. Pánek, Táborský & Čepička.

Diagnosis: Aloricate marine jakobids with acristate mitochondria.

Type genus: *Stygiella* Pánek, Táborský & Čepička, 2014.

Other genus: *Velundella* gen. nov. Pánek, Táborský & Čepička, 2014.

Zoobank registration: urn:lsid:zoobank.org:act:4A238129-B037-4B4E-9D15-E205E7B13605.

***Stygiella* gen. nov. Pánek, Táborský & Čepička**.

Diagnosis: Stygiellidae with grooved cells usually 6–9 μm long, crescent-shaped (in lateral view), with a broadly open, diamond-shaped feeding groove. The groove occupies entire or almost entire ventral side of the grooved cell and reached its posterior end.

Type species: *Jakoba incarcerata* Bernard Simpson & Patterson, 2000 (= *Andalucia incarcerata*, *Stygiella incarcerata*).

Etymology: N.L. fem. dim. n. *Stygiella*, named after the goddess of the river Styx. This river formed a boundary between the Earth and the Underworld.

Zoobank registration: urn:lsid:zoobank.org:act:9EA9ADF7-11C7-40D7-925F-7845D48420C0.

***Stygiella incarcerata* (Bernard, Simpson & Patterson, 2000) comb. nov.**

Synonyms: *Jakoba incarcerata* Bernard, Simpson & Patterson, 2000; *Andalucia incarcerata* (Bernard, Simpson & Patterson, 2000).

Description: grooved cells 6.7–9.5 μm long, often attached to the substrate by the anterior flagellum. Swimming cells relatively rare.

***Stygiella adhaerens* sp. nov. Pánek, Táborský & Čepička**.

Diagnosis: *Stygiella* with grooved cells 5.0–8.4 μm long. Grooved cells very often adhered to the substrate by the anterior or the posterior flagellum and swam rarely.

Syntype: Protargol preparation of the isolate PETROCHORI, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue number 12/96. Fig. 1Z, AA is image from the syntype.

Type locality: Pilos, Petrochori, Greece (36°58'N, 21°39'E).

Habitat: Anoxic marine sediment.

Etymology: L. part. adj. *adhaerens* (adhering).

Gene sequence: SSU rDNA sequence from the strain PETROCHORI, GenBank accession number KP144401.

Zoobank registration: urn:lsid:zoobank.org:act:E2EF3439-3683-42F1-8985-5A550683F374.

***Stygiella agilis* sp. nov. Pánek, Táborský & Čepička**.

Diagnosis: *Stygiella* with grooved cells 5.6–8.9 μm long. Grooved cells adhered to the substrate by the cell body (laterally or dorsally), swam rarely. Swimming cells were extremely abundant and constituted dominant cell morphotype in the population.

Syntype: Protargol preparations of the isolate AND, deposited in the collection of the Dept. of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 9/30-32 and 10/99, 100. Fig. 1AC is image from the syntype.

Type locality: Elephant beach, Havelock Island, Andaman Islands, India (12º00'N, 92º56'E).

Habitat: Anoxic marine sediment.

Etymology: L. adj. *agilis* (agile).

Gene sequence: SSU rDNA sequence from the strain AND, GenBank accession number KP144396.

Zoobank registration: urn:lsid:zoobank.org:act:A0114EE7-55D7-4960-8B92-E1AC20019774.

***Stygiella cryptica* sp. nov. Pánek, Táborský & Čepička**.

Diagnosis: *Stygiella* with grooved cells 6.4–10.5 μm long. Grooved cells very often adhered to the substrate by the anterior or the posterior flagellum and swam rarely. SSU rRNA possesses distinct helix E23/3 in the hypervariable region V4.

Syntype: Protargol preparations of the isolate PC1, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 5/97 and 6/51, 52. Fig. 1X, Y is image from the syntype.

Type locality: Peggy's Cove, Canada (44º29'N, 63º55'W).

Habitat: Anoxic marine sediment.

Etymology: L. adj. *cryptica* (cryptic).

Gene sequence: SSU rDNA sequence from the strain PC1, GenBank accession number KP144402.

Zoobank registration: urn:lsid:zoobank.org:act:21C5B523-3A4E-405C-A7B4-6507E31F3C1B.

***Velundella* gen. nov. Pánek, Táborský & Čepička**.

Diagnosis: Stygiellidae usually 9–12 μm long. Grooved cells possessed a distinct, spiral feeding groove that did not reach the posterior end of the cell.

Type species: *Velundella trypanoides* sp. nov. Pánek, Táborský & Čepička.

Etymology: N.L. fem. dim. n. *Velundella*, named after the Velvet Underground, an American rock band that represent underground music and is often considered as one of the most important and influential groups of the 1960s.

Zoobank registration: urn:lsid:zoobank.org:act:9472415A-259E-484C-83B2-E0D3B273CD32.

***Velundella trypanoides* sp. nov. Pánek, Táborský & Čepička**.

Diagnosis: *Velundella* 7.8–14.9 μm long, a markedly spiral feeding groove almost reaches the posterior end of the cell. Cells noticeably elongated. Grooved cells broad, with conspicuous groove; groove of serpentine-shaped swimming cells is less apparent and narrower. Virtually all grooved cells attached to the substrate by the cell body or, sometimes, by posterior cytoplasmic projections.

Syntype: Protargol preparations of the strain LUC3N, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 6/27, 28, 84, 85. Fig. 1AD, AE are images from the syntype.

Type locality: Brač island, Croatia (43°17'N, 16°52'E).

Habitat: Anoxic marine sediment.

Etymology: derived from Gr. n. *trypáni* (auger, drill) and L. suffix -*oides* (resembling, similar).

Gene sequence: SSU rDNA sequence from the strain LUC3N, GenBank accession number KP144395.

Zoobank registration: urn:lsid:zoobank.org:act:3879E18C-D7F9-4D48-B0B4-EEF635D39664.

***Velundella nauta* sp. nov. Pánek, Táborský & Čepička**.

Diagnosis: *Velundella* with grooved cells 8.4–11.8 μm long; less spiral and shorter groove than *V. trypanoides*. The majority of grooved cells attached to the substrate by flagella, mainly by the anterior one; bulbous protrusion at the posterior pole of the cell. The posterior

flagellum of the swimming cells leaves the shortened groove approximately in ½ of the cell length.

Syntype: Protargol preparations of the strain BMT, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 9/92, 93. Fig. 1AF, AG is image from the syntype.

Type locality: Balikpapan, Kalimantan, Indonesia (1°20' S, 116°50' E).

Habitat: Anoxic marine sediment.

Etymology: L. masc. n. *nauta* (a sailor).

Gene sequence: SSU rDNA sequence from the strain LUC3N, GenBank accession number KP144392.

Zoobank registration: urn:lsid:zoobank.org:act:3879E18C-D7F9-4D48-B0B4-EEF635D39664.

6. Shrnutí a závěr

V průběhu studia jsem se spolu s dalšími členy týmu Ivana Čepičky podílel na vytvoření sbírky kultur volně žijících anaerobních prvoků, která je dnes svou velikostí i množstvím zastoupených skupin zdaleka největší svého druhu na světě. Poskytuje tak unikátní vhled do světa těchto organismů a umožňuje jejich podrobný výzkum. Jednou z hojně zastoupených skupin v této kolekci kultur je kmen Heterolobosea, ačkoliv jeho zástupci jsou jen velmi vzácně detekováni z prostředí chudých kyslíkem pomocí metod nezávislých na kultivaci.

Celkem jsme získali 48 izolátů anaerobních heteroloboseí pokrývajících jejich celkovou diverzitu lépe než předchozí studie. Byly jsme schopni znovu prozkoumat čtyři již dříve popsané druhy – *Percolomonas descissus*, *Psalteriomonas lanterna*, *Sawyeria marylandensis* a *Monopylocystis visvesvarai*. Navíc jsme nově objevili a popsali 11 druhů. S výjimkou tří druhů (*Lyromonas vulgaris*, *Vahlkampfia anaerobica* a *Pleurostomum flabellatum*) tak naše sbírka obsahuje kompletní soubor všech známých obligátně anaerobních heteroloboseí. Rozdělení izolátů do jednotlivých druhů jsme provedli na základě jejich podrobné morfologické charakterizace při současné znalosti jejich fylogeneze na základě analýzy SSU rDNA. Jako pomocné kritérium nám sloužilo porovnání jejich vzájemných genetických vzdáleností (p-distance sekvencí SSU rDNA po odstranění intronů).

Analýza SSU rDNA také jasně ukázala, že drtivá většina všech druhů obligátně anaerobních heterolobosei patří do jediné monofyletické skupiny, Psalteriomonadidae. Jeden izolát představuje nezávisle vzniklou obligátně anaerobní linii, čeleď Creneidae. Čeleď Psalteriomonadidae původně obsahovala pouze rod *Psalteriomonas*, dnes ale zahrnuje pět rodů (Pánek *et al.* 2012). Jedním z nich je i nově popsaný rod *Harpagon*, do něhož jsme přesunuli druh *Percolomonas descissus* (dříve Percolomonadidae).

Fylogenetické analýzy založené na SSU rDNA s vysokou statistickou podporou potvrzují monofylii jednotlivých rodů. Pouze v případě rodu *Monopylocystis* je statistická podpora relativně nízká (viz Pánek *et al.* 2014a). Tyto analýzy dále rozdělují Psalteriomonadidae na tři hlavní linie, jejichž vzájemné příbuzenské vztahy zůstávají nejasné – (1) *Sawyeria* a *Psalteriomonas*, (2) *Harpagon* a *Monopylocystis*, (3) *Pseudoharpagon* (Pánek *et al.* 2014a).

Na základě morfologických znaků jsme do Psalteriomonadidae zařadili i druhy *Lyromonas vulgaris* a *Vahlkampfia anaerobica*, jejichž kultury už sice neexistují a sekvenční data z nich nebyla nikdy získána, nicméně na základě morfologických znaků jde o druhy rodu *Psalteriomonas* a *Monopylocystis*. Celkem tak Psalteriomonadidae v současnosti zahrnují 16 druhů (Pánek *et al.* 2012, 2014a).

Psalteriomonadidae dnes díky našemu výzkumu představují, pokud jde o druhovou diverzitu a počet známých izolátů, jednu z nejprozkoumanějších čeledí heterolobosei. Ukázali jsme, že

společný předek čeledi Psalteriomonadidae byl schopen tvorby stádia améby, cysty i bičíkovce. Je pravděpodobné, že centrální roli v životním cyklu psalteriomonadidů, alespoň ancestrálně, hrálo amébovité stádium, a to díky tomu, že je (na rozdíl od bičíkovce) schopné encystace. To naznačuje fakt, že cysty nebyly nikdy pozorovány v kultuře, kde chybělo stádium améby, a to včetně rodu *Harpagon*, u něhož stádium améby pravděpodobně zcela chybí. Je možné, že u linie *Psalteriomonas-Sawyeria* došlo ke ztrátě schopnosti tvorby cysty a u rodu *Sawyeria* snad i ke ztrátě bičíkatého stádia. Izoláty uvnitř některých druhů skupiny Psalteriomonadidae se mezi sebou lišily přítomností určitých životních stádií. Konkrétně se to týkalo druhů *Monopylocystis disparata*, *M. visvesvarai* a *Psalteriomonas lanterna* (Pánek *et al.* 2012, 2014a). Izoláty obou druhů rodu *Monopylocystis* byly přitom kultivovány s použitím stejného živného média. O důvodech těchto odlišností mezi izoláty lze pouze spekulovat a nelze zcela vyloučit ani to, že jde o permanentní ztrátu schopnosti tvořit určité stádium.

Bičíkatý aparát u zástupců čeledi Psalteriomonadidae obsahuje čtyři bičíky (Pánek *et al.* 2012). My jsme objevili, že bičíkovci druhu *Pseudoharpagon longus* mají většinou 5 bičíků v bičíkatém aparátu (Pánek *et al.* 2014a). To je dosti vzácný stav, neboť bičíkovci, kteří mají mastigont s více než dvěma bičíky, mají obvykle nejen u ostatních heterolobosei, ale i eukaryot většinou sudý počet bičíků. Snad to souvisí se semikonzervativním charakterem ciliární transformace.

Pomocí transmisní elektronové mikroskopie jsme prostudovali strukturu bičíkatého aparátu u druhů *Monopylocystis visvesvarai* (Pánek *et al.* 2012), *Pseudoharpagon tertius* a *P. pertyi* (Pánek *et al.* 2014a). Na základě těchto výsledků jsme definovali synapomorfii bičíkatého aparátu čeledi Psalteriomonadidae, totiž harfovitou strukturu, kterou tvoří napojení svazku mikrofilament (MB) na mikrotubulární kořen R2 (Pánek *et al.* 2012). Zavedli jsme novou terminologii mikrotubulárních kořenů dle Yubuki *et al*. (2013) u kmene Heterolobosea (Pánek *et al.* 2014a). Díky tomu lze homologizovat jednotlivé struktury v bičíkatém aparátu heterolobosei s jinými eukaryotickými skupinami.

Pomocí transmisní elektronové mikroskopie jsme prostudovali i morfologii mitochondriálních derivátů u zástupců čeledi Psalteriomonadidae. Souhrně lze říci, že u všech tří druhů rodu *Psalteriomonas* (Broers *et al*. 1990, 1993; Pánek *et al.* 2012), u druhu *Monopylocystis visvesvarai* (O´Kelly *et al*. 2003; Pánek *et al.* 2012), *Harpagon schusteri* (Brugerolle a Simpson 2004) a *Sawyeria marylandensis* (Barberà *et al*. 2011) byly pozorovány výhradně akristátní mitochondriální deriváty. Přechodový stupeň mezi klasickými a akristátními MROs by mohl představovat druh *Pseudoharpagon pertyi*, v jehož MROs jsme pozorovali invaginace vnitřní mitochondriální membrány připomínající individuální diskoidální kristy známé u jiných heterolobosei (Pánek *et al.* 2014a).

Dále jsme objevili a nově popsali čeleď Creneidae zahrnující obligátně anaerobní druh *Creneis carolina* (Pánek *et al.* 2014b). Životní cyklus tohoto druhu se zcela vymyká kanonickému životnímu

cyklu heterolobosei. Jako jediný dosud známý zástupce kmene Heterolobosea totiž *C. carolina* tvoří stádium améby permanentně vybavené bičíkem (tj. améboidního bičíkovce). Navíc jsou buňky tohoto druhu schopny transformace do dalšího neobvyklého stádia, bizarního mnohobičíkovce vybaveného dvěma předními bičíky a 12 bičíky umístěnými po obvodu buňky (Pánek *et al*. 2014b).

Na základě detailního studia morfologie tohoto druhu (včetně ultrastrukturní studie) jsme vyslovili dvě hypotézy o jeho fylogenetické pozici: (1) *Creneis carolina* a Psalteriomonadidae jsou blízce příbuzní (2) *C. carolina* je blízce příbuzná skupině Percolatea. Ani fylogenetické analýzy SSU rDNA, ani analýzy založené na konkatenátu sekvencí SSU rDNA a dvou protein-kódujících genů (gen pro alfa- a beta-tubulin) nedokázaly mezi těmito dvěma hypotézami spolehlivě rozhodnout (Pánek *et al*. 2014b). Až nové dosud nepublikované multigenové analýzy založené až na 132 genech potvrdily, že Creneidae představují nezávisle vzniklou obligátně anaerobní linii heterolobosei, která je blízce příbuzná skupině Percolatea. Je tedy jasné, že obligátně anaerobní způsob života se uvnitř kmene Heterolobosea vyvinul nejméně dvakrát nezávisle na sobě. *Pleurostomum flabellatum* (Tulamoebidae) je potenciálně třetí takovou nezávisle vzniklou linií, ale o anaerobním způsobu života tohoto druhu se ví jen velmi málo (Park *et al*. 2007).

S využitím transmisní elektronové mikroskopie jsme podrobně u druhu *Creneis carolina* prozkoumali buněčnou strukturu stádia améboidního bičíkovce. Jediný bičík v bičíkatém aparátu tohoto stádia se nám podařilo homologizovat s nejstarším bičíkem ostatních eukaryot. Jeho bazální tělísko nese čtyři nemikrotubulární elementy a dva mikrotubulární kořeny, R1 a R2. Identitu R2, respektive R2/I systému jsme potvrdili díky několika specifickým znakům, které sdílí s ostatními zástupci kmene Heterolobosea i jinými eukaryoty. Z našich dat ale zároveň plyne, že R2/I systém, nebo dokonce celý bičíkatý aparát *C. carolina*, prošel reverzí chirality oproti tomu, co známe u jiných linií. Alternativním vysvětlením tohoto jevu je hypotéza, že reverzí chirality prošel bičík a jeho bazální tělísko. Ať už platí jedna nebo druhá hypotéza, jde o dosud zřejmě bezprecedentní změnu ve vývoji bičíkatého aparátu eukaryot. R2/I systém lze u *C. carolina* na základě několika unikátních charakteristik jasně identifikovat – (1) mikrotubulární kořen R2 je asociován s fibrilou připomínající svou mřížovanou stukturou I fibrilu ostatních heterolobosei; (2) na R2 se připojuje žíhaná fibrila připomínající rhizoplast; (3) R2 je rozděleno na dvě části a (4) na cytoplasmatické straně R2 je část mikrotubulů podložena vzájemně propojenými paprsky, které jsme předtím pozorovali na R2 i u druhu *Pseudoharpagon pertyi* (Pánek *et al*. 2014a, 2014b).

Reverzi chirality naopak naznačují poziční charakteristiky R2/I systému - R2 u *Creneis carolina* vzniká na levé straně bazálního tělíska (nikoliv na pravé); rhizoplast vzniká na pravé straně R2 (nikoliv na levé); je to vnitřní část R2 (R2a), která vede dolů na pravé straně buňky, kdežto u Psalteriomonadidae u rodu *Pleurostomum* tuto stranu buňky podporuje vnější část R2. R2

identifikovaný u *C. carolina* tak velmi připomíná R2 ostatních heterolobosei, až na to, že je jeho zrcadlovým obrazem.

Protože jsou anaerobní heterolobosea jen velmi málo zastoupena v environmentálních knihovnách, nelze odhadnout, jak velkou část diverzity je možné pomocí kultivačního přístupu odhalit. Určitým vodítkem při odhadu efektivity jednotlivých přístupů při odhalování diverzity může být naše studie zaměřená na diverzitu anaerobních jakobidů. Sekvence patřící jakobidům jsou často nalézány v environmentálních knihovnách z anoxických habitatů, ačkoliv kultivační přístup odhalil doposud jen jediný druh, *Andalucia incarcerata* (Simpson a Patterson 2001). My jsme získali 21 nových izolátů anaerobních jakobidů představujících 6 druhů a detailně jsme prohledali všechny dostupné environmentální knihovny z anoxických a mikrooxických prostředí (712 sekvencí). Zjistili jsme, že oba přístupy dohromady odhalily 10 různých druhů jakobidů. Ty tvoří monofyletickou linii, Stygiellidae, která je významnou složkou mikrobiálních komunit v anoxických mořských sedimentech bohatých na amoniak a sulfan (Pánek *et al*., připraveno k zaslání do tisku). Zajímavé je, že kultivační přístupy byly schopné odhalit 60 % všech existujících druhů, to znamená, že úspěšnost kultivačních metod je v tomto případě výrazně vyšší, než bychom čekali například při porovnání se situací u bakterií, kde je kultivovatelných zhruba 1 % druhů (Pham a Kim 2012; Rappé a Giovannoni 2003).

Při zobecnění těchto poznatků na Heterolobosea je ale samozřejmě potřeba brát v úvahu, že námi používaná kultivační média jsou vhodná jen pro pěstování anaerobních druhů, které pro svůj růst nevyžadují extrémní hodnoty salinity, acidity a dalších faktorů. Je proto možné, že v extrémních typech habitatů se doposud skrývají další, doposud neobjevené anaerobní linie heterolobosei. Příkladem, který to ilustruje, je nejen *Pleurostomum flabellatum* obývající hypersalinní prostředí (Park *et al*. 2007, Park a Simpson 2015), ale i linie čeledi Psalteriomonadidae blízce příbuzná rodům *Sawyeria* a *Psalteriomonas*, která byla zachycena environmentálními přístupy v extrémně kyselém prostředí Río Tinto (Amaral-Zettler *et al*. 2011; Pánek *et al*. 2012).

Naše výsledky poskytly pevné základy výzkumu diverzity anaerobních heterolobosei, odhalily jejich velkou a doposud neznámou druhovou diverzitu a stanovily základní rámec jejich klasifikace. Ukázali jsme, že existují minimálně dvě nezávislé obligátně anaerobní linie heterolobosei, což při vědomí toho, že některé další linie jsou pravděpodobně fakultativně anaerobní, činí z této skupiny ideální model pro studium evoluce anaerobiózy. Předběžná analýza transkriptomických a genomických dat ukázala, že Heterolobosea již od raných fází svého vývoje disponovala některými enzymy umožňujícími efektivnější energetický metabolismus v anoxickém a mikrooxickém prostředí (Ppi-PFK a PPDK) a enzymy, které jsou důležitou součástí anaerobního metabolismu pyruvátu u anaerobních prvoků (HydA, HydE, HydF, HydG). Další klíčové enzymy energetického metabolismu obligátně anaerobních heterolobosei pak byly získány horizontálním genovým přenosem zřejmě až při vzniku obligátně či fakultativně anaerobních linií. Dobrým příkladem takového genového přenosu

je ACS, které má u rodů *Creneis* a *Naegleria* jasně odlišný evoluční původ. Podobně lze uvažovat i o genu pro PFO u *Creneis* a Psalteriomonadidae. Není bez zajímavosti, že předpokládaným zdrojem obou těchto horizontálně přenesených genů u *Creneis* jsou zástupci linie Fornicata.

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